SOME OBSERVATIONS ON BEEF CATTLE AFFECTED WITH GENERALIZED EDEMA OR ANASARCA DUE TO VITAMIN A DEFICIENCY

LOUIS L. MADSEN[1] AND I. P. EARLE
WITH THE TECHNICAL ASSISTANCE OF RUSSELL E. DAVIS, HARRY BASTRON, C. W. THIES, CHARLES A. CABELL AND H. DEAN RAY
Animal Husbandry Division, Bureau of Animal Industry, United States Department of Agriculture, Beltsville, Maryland

ONE FIGURE

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The fact that prolonged vitamin A deficiency in cattle may result in edema as characterized by swelling of the legs, shoulders and brisket, on the abdomen, in the hindquarters and elsewhere has been recognized for some time. This condition is often referred to as anasarca. Hastings ('41, '44), Cady ('42) and others have more recently called attention, in the popular press, to anasarca in fattening cattle apparently due to vitamin A deficiency in rations of which the grain consisted principally of yellow corn. Similar cases have been brought to the attention of the Bureau of Animal Industry when distillery slop and low-quality roughages have been fed to fattening cattle.

In 1941 our attention (Creech and Madsen, '42; Mohler, '42; and Madsen, '42) was called to a condition characterized by extensive edema of the subcutaneous adipose tissue and musculature of beef carcasses which was encountered by

[1] Now Head, Department of Animal Husbandry, Utah State Agricultural College.

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Federal meat inspectors at several meat packing establishments. In view of the unusually large number of cases of unrecognized origin (see table 1) the senior author was called to the Corn Belt area where a number of these cattle had been fed, to determine if a nutritional basis could be established for the disease since previous bacteriological studies had been entirely negative.

TABLE 1

Beef carcasses condemned for anasarca by months from July, 1941 to December, 1946, inclusive.

	YEAR						
	1941	1942	1943	1944	1945	1946	Monthly average
January	..	2	3	9	5	5	5
February	..	3	11	5	2	12	7
March	..	9	4	4	3	9	6
April	..	6	5	5	5	10	6
May	..	9	10	7	9	6	8
June	..	5	15	9	11	5	9
July	3	12	18	18	13	10	12
August	18	5	20	3	9	8	10
September	40	6	30	3	11	3	15
October	43	9	28	5	5	7	16
November	20	4	13	7	11	10	11
December	15	5	12	6	6	12	9
Yearly total	139	75	169	81	90	97	

The present paper reports statistics on the number of beef carcasses condemned for anasarca by the Federal meat inspectors during the years 1941–1946, and presents data on the occurrence, clinical appearance and history of field cases of anasarca as they were observed during the field trip to the Corn Belt in 1941. It also describes the experimental production of edema in vitamin A-deficient cattle and presents results of observations made on the blood chemistry in both field cases of anasarca and experimentally produced cases of this condition.

CARCASSES CONDEMNED

The data given here on condemned carcasses were furnished through the courtesy of the Meat Inspection Division of the Bureau. The records show that 651 carcasses were condemned for anasarca by Federal meat inspectors from July, 1941 through December, 1946. The distribution of these cases on a monthly and yearly basis is given in table 1. Heavy losses were encountered in the last half of 1941. Fewer cases were condemned in 1942 but losses were heavy in 1943. About the same number were condemned in 1945 as in 1946. It appears likely that the years of relatively few numbers of anasarca cases are related to the prevailing light or short-time grain feeding of steers. From these limited data it seems that more cases appear in the latter half of the year. These statistics do not represent the total loss among slaughter animals due to anasarca since in cases in which the edema is localized the carcass may be retained, trimmed as found necessary under inspection and released.

FIELD CASES OF ANASARCA

Statistics are not available on the number of cases of anasarca that develop under practical conditions. It is obvious, however, that more cases develop than are condemned at slaughter since some cases recover before slaughter because of timely changes in the ration, whereas others may die on the farm or become severely injured during convulsions while in transit. Seven farms having approximately 1200 cattle on feed were visited in the Corn Belt area by the senior author in company with Dr. John S. Koen and Dr. H. J. Classick, both veterinarians of the Bureau of Animal Industry on field assignments. Case reports from 2 of these farms are presented to illustrate (1) the development of vitamin A deficiency associated with anasarca under ordinary feed lot conditions and (2) a means of preventing serious losses by simple corrective feeding.

Case 1. Mr. G. started feeding 41 head of yearling Hereford steers, obtained from the West, in November, 1940. They were

started on feed with corn and fodder of the 1940 crop and about 1 pound per head per day of a mixture of equal parts molasses and alfalfa which was continued until June, 1941. When the hot weather came during the latter part of June and persisted through July and August the cattle "went off feed," but no edema was seen by the owner. At this time feeding alfalfa hay of the 1940 crop 3 times daily improved the appetite of the steers and they were soon brought back to full-feed. Early in September oat straw was substituted for the alfalfa hay. Straw feeding was continued until early in December when Dr. Koen visited the farm and suggested a change in roughage from oat straw to alfalfa on the basis of information he had received from the Bureau that anasarca may be due to vitamin A deficiency. At this time about 30 animals of the group had swollen legs and enlarged briskets. One steer could hardly walk and 1 animal had recently been shipped to Omaha and condemned for anasarca. We visited the farm on December 18, 1941, about 2 weeks after alfalfa feeding was started, and according to Dr. Koen improvement in the cattle was evident. Dr. Koen and Dr. Classick visited this farm again on January 21, 1942, and found so much improvement in the cattle that little or no edema could be observed. All of the cattle were sold about 1 week later, or approximately 8 weeks after alfalfa feeding was started, without evidence of anasarca.

Case 2. On this farm 135 yearling steers were started on feed in October, 1940. The animals were turned into a corn field where they had access to corn stalks and some blue-grass until January, 1941. At this time they were taken up to the feed lot and given Atlas sorghum silage at about 30 pounds per head daily and all the ground yellow corn (1940 crop) they would clean up. A small amount of soybean hay was also given until the supply was used, about June 1, 1941. At this time feeding of oat hay was started and corn silage was fed until August. After all the silage had been consumed, oat hay was continued as the only roughage. On November 6, 1941,

20 of the steers were shipped to Omaha, and of this group 1 was condemned for anasarca. A few more animals were sent to Chicago about the middle of November and 1 of these was also condemned for anasarca. Another steer became very lame and a veterinarian advised that the animal had probably sprained its leg, and that the steer should be killed and used for food. The steer was butchered but the carcass was declared unfit for food owing to generalized edema.

When we visited the farm on December 19, 1941, several animals showed marked symptoms of vitamin A deficiency. One animal was blind and had swollen legs and brisket. This animal was down and died the following day. Two other steers were also markedly affected, 1 was blind and another had severe edema of the legs, brisket and shoulders.

Twelve days previous to our visit Dr. Koen had seen these cattle and suggested feeding alfalfa as in case 1. This had resulted in a marked improvement in appetite and general appearance of the steers. The foreman commented that the cattle could get up and go to the feed bunks with greater ease since they had been receiving alfalfa, and that the lameness which had been previously noted had now practically disappeared. The foreman also characterized as "fish-eyes" the peculiar protruding eyes of several of the cattle, a condition which is typical of vitamin A-deficiency blindness.

The findings presented in these 2 cases were typical of conditions noted on other farms where cases of anasarca were seen. In some instances other symptoms of vitamin A deficiency such as diarrhea, constipation, nightblindness, stiff gait and convulsions were prominent. In 1 instance impaired vision in the cattle was not suspected until difficulty was encountered in loading the cattle on a truck. On this farm the vision of an apparently blind steer improved remarkably after alfalfa hay had been added to the ration in place of oat hay. In some feed lots anasarca developed in cattle fed stored corn, but in these cases a low-carotene roughage was also used.

EXPERIMENTAL CASES OF ANASARCA

Experimental cases of anasarca were produced by feeding either a carotene-deficient ration (diet 1) or one containing a large amount of yellow corn (diet 2), the roughage in both cases being supplied by oat straw. The concentrate portion of diet 1 had the following percentage composition: dried beet pulp, 45; cracked white corn, 40; linseed meal, 7; soybean meal, 7; steamed bonemeal, 0.5; and salt, 0.5. The concentrate portion of diet 2 consisted, in per cent, of cracked yellow corn, 80; dried beet pulp, 8.5; linseed meal, 10; steamed bonemeal, 1; and salt, 0.5. Vitamin A-deficiency edema was readily produced when cattle were restricted to either of these rations for several months. In a number of instances, after the appearance of anasarca, the diet was supplemented with 1 of several sources of carotene and vitamin A such as alfalfa leaf meal, carotene concentrate, crystalline carotene, cod-liver oil and distilled vitamin A concentrate in natural ester form. In all such cases, anasarca, as well as other symptoms of vitamin A deficiency except permanent blindness, was entirely relieved with the treatment.

A complete history is given for only 1 case (steer 411) in which the course of depletion and the development of deficiency symptoms were quite characteristic of all the other cases. Since a significant part of the story is believed to be told by the results of the blood analyses which were made at approximately monthly intervals on a number of these edema cases, these data are included in the history, although the general subject of blood analyses in these cattle is treated in a later section of this paper.

Steer 411 on which the case history of experimental edema is based was fed individually on diet 2 (80% yellow corn). This animal was a purebred Shorthorn approximately 7 months old weighing 613 pounds at the beginning of the experiment. Feed consumption, live-weight gain, together with the appearance of progressive symptoms of convulsions, diarrhea, blindness and edema are indicated in table 2 and illustrated in figure 1. Attention is called to the fact that

TABLE 2

Summary of observations on steer no. 411 while receiving an 80% yellow corn grain ration.

OBSERVATION	DAYS OF EXPERIMENT											
	27	58	90	117	147	177	209	238	271	289	300	328 [1]
Condition of animal	Normal	Normal	Normal	Normal	Normal	Convulsions	[2]	Diarrhea	Blind	Edema	[3]	[3]
Body weight, lbs.	635	675	712	765	827	873	921	970	1033	1056	1062	1069
Average daily gain for period, lbs.	1.44	1.29	1.13	1.96	2.07	1.53	1.50	1.69	1.91	1.28	0.55	0.25
Daily feed consumption:												
Grain mixture, lbs.	7.7	8.9	10.8	11.9	12.1	13.9	14.6	15.1	15.7	16.0	17.9	14.4
Oat straw, lbs.	2.2	1.6	1.8	2.2	2.2	2.0	2.0	1.2	1.0	1.7	1.3	0.7
Plasma nitrogen fractions:												
Total plasma nitrogen, gm/100 ml	1.019	0.993	1.046	1.032	1.107	1.105	1.007	1.051	1.019	1.071	1.089	1.167
Nonprotein nitrogen, gm/100 ml	0.022	0.041	0.048	0.039	0.061	0.041	0.028	0.029	0.025	0.022	0.042	0.024
Fibrinogen nitrogen, gm/100 ml	0.052	0.060	0.088	0.063	0.074	0.060	0.055	0.075	0.064	0.133	0.142	0.133
Euglobulin nitrogen, gm/100 ml	0.089	0.081	0.082	0.081	0.106	0.076	0.119	0.096	0.081	0.083	0.085	0.150
Pseudoglobulin nitrogen, gm/100 ml	0.283	0.288	0.308	0.320	0.320	0.310	0.270	0.309	0.319	0.362	0.363	0.423
Total globulin nitrogen, gm/100 ml	0.424	0.429	0.478	0.464	0.500	0.446	0.444	0.480	0.464	0.578	0.590	0.706
Albumin nitrogen, gm/100 ml	0.573	0.523	0.520	0.529	0.546	0.618	0.535	0.542	0.530	0.471	0.457	0.437
A/G ratio	1.35	1.22	1.09	1.14	1.09	1.39	1.20	1.13	1.14	0.81	0.77	0.62
Plasma:												
Carotene, μg/100 ml	86	35	32	33	46	39	38	40	46	....	27	21
Vitamin A, μg/100 ml	25	16	23	11	13	13	9	10	7	....	8	6
Vitamin C, mg/100 ml	0.41	....	....	0.56	0.54	0.40	0.39	0.43	0.34	....	0.31	0.33
Serum:												
Calcium, mg/100 ml	....	....	13.1	10.6	11.0	12.0	10.9	10.2	11.1	....	11.5	9.8
Magnesium, mg/100 ml	2.8	....	2.6	2.6	2.4	2.5	2.4	2.3	2.3	....	2.7	2.3
Inorganic phosphorus, mg/100 ml	....	7.8	8.1	8.2	7.6	7.7	6.1	6.9	7.2	5.8	6.3	4.8
Phosphatase activity (Bodansky units)	....	4.5	3.1	4.9	4.6	4.2	3.8	4.6	4.1	4.4	3.3	3.1

[1] Steer died during a convulsion on the 343rd day.
[2] Beginning stages of rough hair coat, slobbering and slight edema.
[3] Edema increasing rapidly; convulsions more numerous. Ascorbic acid therapy started 303rd day.

there was an increase in grain consumed up to the three hundredth day, but that feed consumption and rate of gain decreased after this time. No blood analyses were made after the three hundred and twenty-eighth day. Typical changes in the plasma protein fractions were observed at about the same time that edema appeared. Plasma carotene, vitamin A and

Fig. 1 These photographs illustrate symptoms of vitamin A deficiency in steer 411 after 309 days on diet 2. A. The steer is blind and has a rough, dry hair coat, but has made fairly good gains in weight. B. The hind legs are swollen due to edema. C. Rapid respiration was common and even panting occurred on slight exertion in hot weather. Note slobbering. D. Convulsions occurred frequently. The head is drawn back typically. Note left horn fracture (C) received during previous convulsions.

vitamin C decreased in the usual manner, but there were minor fluctuations in these during the experiment. Serum calcium, inorganic phosphorus and phosphatase activity tended to decrease but the changes were not marked until after about the three hundredth day at which time the food intake also decreased.

The highest average grain consumption for steer 411 was recorded for the period between the two hundred and eighty-ninth and three hundredth days. An average of 17.9 pounds of grain mixture or 14.3 pounds of yellow corn was eaten daily at this time. On analysis the corn was found to contain 2.1 μg of crude carotene per gram and on biological assay with rats a value of 1.6 International Units (I.U.) of vitamin A per gram was obtained. At the above level of yellow corn consumption, and using the assay figures just mentioned, the steer received 13.6 mg of crude carotene daily, or 10,378 I.U. of vitamin A. This amount of carotene from yellow corn was insufficient to alter the course of vitamin A depletion. Feed consumption declined, edema increased and the animal died in convulsion on the three hundred and forty-third day of the experiment. Another steer on the same ration died in convulsion on the two hundred and forty-ninth day of the experiment. In this case edema appeared about the two hundred and thirteenth day, and some impairment of day vision was evident before death.

These findings add further evidence to the field observations that vitamin A-deficiency symptoms together with edema develop in fattening cattle when their ration is made up of yellow corn, a protein supplement and a low-carotene roughage.

BLOOD ANALYSES

Before attention was directed to the field cases of anasarca occurring in the Corn Belt, some data were already available on the composition of blood from a number of experimental animals both under conditions of apparently normal health and also at various stages of vitamin A deficiency which developed during prolonged use of diet 1. These data seemed

to indicate that there were certain characteristic changes in the composition of the blood, particularly with respect to the protein fractions, approximately coincident with the development of edema in these vitamin A-deficient animals. Analyses of blood obtained from some field cases of anasarca were undertaken primarily for the purpose of further characterization of the general physiological condition of the animals and also for the further identification of this condition with the edema occurring in the experimental animals depleted of vitamin A. Results of analyses of blood from the field cases seemed to indicate that changes had occurred here similar to those in the experimental edematous animals. In view of the feeding practices under which the anasarca cases observed had developed in the Corn Belt, blood studies were also made on animals which were fed diet 2 containing 80% yellow corn.

Blood samples for analysis were drawn from a jugular vein. For the preparation of plasma samples, exactly 30 mg of neutral potassium oxalate were used as anticoagulant for each 10 ml of blood. Plasma proteins were fractioned by Howe's ('21) micro-method with the variation later recommended by him ('23) that 0.75 molar sodium sulfate be used for the precipitation of fibrinogen in plasma instead of calcium chloride. All nitrogen determinations were made on aliquots of the filtrates by a micro-Kjeldahl method. The spectrophotometric method previously described by Madsen and Davis ('38) and Davis and Madsen ('41) was used for determination of plasma carotene and vitamin A. Plasma vitamin C was determined by the method of Mindlin and Butler ('38). Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method as described by Hawk and Bergeim ('37); serum magnesium was precipitated according to the method used by Godden ('37) and determined as phosphorus by the method of Bodansky ('32). Bodansky's ('33) methods were also used for the determination of serum inorganic phosphorus and serum phosphatase activity.

RESULTS

The results of analyses on cases of experimental edema produced in 7 beef animals have been averaged and paired with the average of observations made on these same animals when they were normal. The averages are presented in table 3 together with the average results of blood analyses made on 5 field cases of anasarca.

It is obvious from the figures presented in table 3 that the plasma protein fractions in the experimental animals with edema differ significantly from those in the same animals

TABLE 3

Summary of results of blood analyses from field and experimental cases of anasarca and normal beef animals.

CONSTITUENTS	AVERAGE OF 5 FIELD CASES OF ANASARCA	EXPERIMENTAL CASES		
		Averages of 7 with edema	Averages of 7 when normal	Calculated odds — edematous vs. normal [1]
Nitrogen fractions in plasma:				
Total plasma nitrogen, gm/100 ml	1.263	1.150	1.076	104
Non-protein nitrogen, gm/100 ml	0.038	0.036	0.037	
Fibrinogen nitrogen, gm/100 ml	0.151	0.150	0.061	191
Euglobulin nitrogen, gm/100 ml	0.166	0.180	0.136	51
Pseudoglobulin nitrogen, gm/100 ml	0.494	0.384	0.318	1999
Total globulin nitrogen, gm/100 ml (including fibrinogen)	0.811	0.714	0.515	2499
Albumin nitrogen, gm/100 ml	0.414	0.400	0.524	587
A/G ratio	0.51	0.56	1.02	
Vitamins in plasma:				
Carotene, μg/100 ml	31.	21	67	26
Vitamin A, μg/100 ml	8	7	23	9999
Vitamin C, mg/100 ml	..	0.25 [2]	0.37 [2]	13
Constituents in serum:				
Calcium, mg/100 ml	..	10.01	11.00	160
Magnesium, mg/100 ml	..	2.63	2.72	
Inorganic phosphorus, mg/100 ml	..	5.60	6.74	40
Phosphatase activity (Bodansky units)	..	2.28	4.24	232

[1] Student's method used for estimating significance between means of paired observations in experimental cases of anasarca and corresponding normal control values.

[2] Average for only 4 animals.

when normal. Attention is called to the increases in fibrinogen and total globulin and to the decrease in albumin in the animals with edema.

Low values for plasma carotene and vitamin A were observed in all cases of anasarca. Calculated odds for estimating the significance of differences in the pairs of average values for plasma carotene and for vitamin A were greater for vitamin A than for carotene. This may be explained by the uniformly low values for vitamin A and carotene in all cases of anasarca as contrasted with uniformly high values for vitamin A and high, but variable, values for carotene in normal animals. The carotene intake of the normal cattle was not standardized, but in all cases was above a deficiency level. In the field cases of anasarca the average blood carotene and vitamin A were higher than in the experimental cases, a condition which is probably due to the fact that alfalfa hay feeding had already been started in some of these animals when blood samples were taken.

Average content of vitamin C in plasma was found to be lower while edema was present than when it was absent in the same animals. The significance of the difference in the 2 averages is shown by the calculated odds not to be great, however, probably because data for this comparison were available from only 4 pairs of observations and because the degree of vitamin A deficiency was not the same in all the animals when the observations were made. In the 4 cases observed there was always a decrease in plasma vitamin C as the deficiency of vitamin A progressed but the drop was not uniform in all cases. This decrease in plasma vitamin C in cases of vitamin A deficiency was first demonstrated by Phillips and coworkers ('38).

While serum calcium, magnesium and inorganic phosphorus tended to decrease slightly from the levels observed when the same animals were normal, the values still remained well within the normal range. There was, however, a decrease from normal in phosphatase activity of the serum in all cases of experimental anasarca studied.

DISCUSSION

Losses due to anasarca associated with vitamin A deficiency are economically important. Nevertheless they can be prevented by proper feeding. Supplying fattening cattle with a small amount of well-cured alfalfa hay successfully avoided serious losses in 2 instances cited. Any other well-cured hay having a good green color, well-made silage, green pasture or a marine oil such as cod-liver oil would no doubt have accomplished the same purpose. The prevention of anasarca or of other symptoms of vitamin A deficiency by the use of proper feeds does not necessarily discourage the highly economical practice of feeding low-carotene roughages such as ordinary cereal hay, straw and corn stover, etc. When these roughages are used, however, it is necessary to supply a supplementary ration of well-cured green hay to avoid possible vitamin A depletion in the latter part of the feeding period.

Opinions have been expressed in the literature and have been prevalent among some cattle feeders that anasarca is caused by feeding stored corn. In both field and experimental cases of anasarca presented in this paper, however, yellow corn of the current season's crop failed to prevent the development of either anasarca or other symptoms of vitamin A deficiency. This observation confirms the report by Hastings ('44). The real error in practical feeding appears to be not in the use of old corn but in the exclusive use of low-carotene roughages such as oat hay and straw and the failure to supplement these low-carotene roughages with green hay less than 1 year of age. Average yellow corn usually contains no more and often less carotene than a low grade hay. Corn should not be depended upon to supply enough carotene to satisfy the vitamin A needs of cattle.

The clinical symptoms and blood findings presented suggest that the field cases and experimental cases of anasarca probably arose from the same condition. All cases of anasarca observed evidenced low levels of blood carotene and vitamin A and, in addition, showed the same changes in blood protein

fractions. These changes consisted essentially of an increased level of total globulins, due principally to increased fibrinogen and, to a lesser degree, to increases in the euglobulin and pseudoglobulin fractions. At the same time there was a definite, although not in every case large, decrease in plasma albumin. The total protein was usually slightly elevated when edema was present.

The relation of the altered protein picture to vitamin A deficiency is not apparent. Increases in fibrinogen in conditions in which destruction and inflammation of tissues occur and increases in other globulin fractions, either with or without increased fibrinogen, in cases of chronic infections, are usually accompanied by some reduction in albumin concentration, an effect presumably secondary to the increase in globulin. Since a reduction in blood albumin is a primary factor in some types of edema, the question arises as to the relation between the lowered albumin and the development of edema in these cattle. The concept of an increased tendency toward transudation and accumulation of fluid in the interstitial tissues with a lowering of the plasma colloid osmotic pressure is a familiar one. In this relation, the concentration of albumin is of greater significance than that of globulins, since the albumin exerts about 2.4 times as great an osmotic pressure as the globulins. It is estimated that at the so-called critical levels for albumin nitrogen and for total plasma nitrogen in the human (i.e., levels below which edema usually appears), the plasma colloid osmotic pressure is reduced to roughly two-thirds of the normal level (Moore and Van Slyke, '30).

If it is assumed that approximately similar relative levels for albumin and for total protein are critical for cattle, it becomes apparent that the average reduction in albumin observed in the anasarca animals is not great enough to account for the appearance of edema, especially in view of the increase in total protein. Values for colloid osmotic pressure calculated by application of the formula of Wells, Youmans and Miller ('33) to data obtained on concentration of proteins in

serum of these animals (unpublished data), also suggest that increases in globulins are great enough to compensate in large measure for the reduction in albumin. It is believed, therefore, that the occurrence of edema in these animals is not entirely related to a lowering of the colloid osmotic pressure of the plasma brought about by a lowering of the albumin concentration.

SUMMARY

Statistics are presented showing that 651 beef carcasses were condemned for generalized edema or anasarca by Federal meat inspectors during the period of July, 1941 to December, 1946, inclusive. Other symptoms of vitamin A deficiency were found to be prevalent among cattle with anasarca in the Corn Belt area. This edematous condition was observed to occur in cattle after a long fattening period in dry lot when fed either stored or new corn in combination with a roughage of low carotene content such as oat hay or straw. Alfalfa hay was highly effective in curing the condition. It is concluded, therefore, that the major dietary error in the production of this deficiency disease is the exclusive use of low-carotene roughages throughout the feeding period rather than the use of old yellow corn.

Cases of anasarca were readily produced experimentally by feeding a carotene-deficient ration or a grain ration containing 80% of new-crop yellow corn together with oat straw as roughage.

Results of blood studies made on field and experimental cases of anasarca produced by vitamin A deficiency indicate a marked similarity in the 2 conditions. Affected animals showed: (1) deficiency levels of blood plasma vitamin A and carotene and a decrease in plasma vitamin C as the vitamin A deficiency progressed, (2) an increase in total plasma globulin due usually to a marked increase in plasma fibrinogen with smaller increases in the other globulin fractions, (3) a decrease in plasma albumin, and (4) an increase in total plasma nitrogen.

In the serum of affected cattle, calcium and inorganic phosphorus decreased slightly; phosphatase activity was reduced while magnesium was relatively unchanged.

It is pointed out that the edema of vitamin A deficiency in cattle appears not to be entirely related to a lowered colloid osmotic pressure of the plasma through reduction in the albumin, since the increases in other protein fractions are estimated to compensate largely in this respect for the decrease in albumin.

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THE FOLIC ACID REQUIREMENT OF TURKEY POULTS ON A PURIFIED DIET [1, 2, 3]

WALTER C. RUSSELL, M. WIGHT TAYLOR AND JAMES V. DERBY, JR [4]
Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey

TWO FIGURES

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The deficiency syndrome in turkey poults, caused by a lack of vitamin B_c, now known to be a form of pteroylglutamic acid, was first described by Richardson, Hogan and Kempster ('45). Growth failure, cervical paralysis, and lowered red cell volume were the principal deficiency signs. Folic acid (pteroylglutamic acid) deficiency in turkey poults has more recently been described by Jukes and associates ('47).

The present paper is concerned with the determination of the folic acid level necessary for full growth, normal hemoglobin production and normal feathering of the turkey poult fed purified diets which differ in their protein content.

EXPERIMENTAL

One hundred and two turkey poults which had been fed a starting mash for the first 8 days of life, were distributed in 11 groups according to weight and color type, so that each

[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Agricultural Biochemistry.

[2] This study was made possible by a grant from Lederle Laboratories, Inc., Pearl River, New York.

[3] Presented before the Division of Biological Chemistry, American Chemical Society, Atlantic City, New Jersey, April 14–18, 1947.

[4] Present address: Patuxent Research Refuge, U. S. Department of the Interior, Bowie, Maryland.

group contained 10 birds, except that groups 8 and 9, which received no folic acid, were allotted only 6 poults each. The 4 color types which comprised the hatch were distributed so that each group contained 5 or 6 poults of the Jersey buff color type and 1 or 2 each of the black, barred, or white types, to make a total of 10.

The 2 purified diets differed in their contents of casein, gelatin and starch but contained the same quantities in grams per 100 gm of diet, of the following ingredients: sucrose, 10; corn oil, 10; mineral salts [5], 5; $CaHPO_4$, 2.0; $Ca\ CO_3$, 0.4; cod liver oil (2,000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram), 0.7; choline chloride 0.2; inositol, 0.1; and para-aminobenzoic acid, 0.1. The following quantities of additional vitamins were provided per 100 gm of diet: thiamine, 1 mg; pyridoxine, 1 mg; riboflavin, 2 mg; calcium pantothenate, 2 mg; nicotinic acid, 5 mg; 2-methyl-1, 4 naphthoquinone, 2.5 mg; mixed tocopherols, 2.5 mg; and biotin, 10 μg.

In an earlier experiment, poults which consumed a purified diet in which 43% protein was supplied by casein (37%) and gelatin (10.6%) grew better than those on a starting mash, made of commonly used ingredients, containing 25% protein. For the present experiment these same diets were used, and in addition a purified diet containing 25% protein supplied by casein and gelatin, in the same ratio as that used for the diet containing 43% protein, so that a comparison could be made of the results, at the same protein level, on the purified diet and on the starting mash.

Diet A (25% protein) was prepared by adding 21.4 gm of casein (alcohol extracted), 6.1 gm of gelatin, and 44 gm of starch to the above ingredients to make 100 gm.

[5] The salt mixture, devised by A. G. Hogan (personal communication), had the following composition and was modified only by doubling the quantity of manganese. The quantities are expressed in grams. $CaCO_3$, 267; $CaHPO_4 \cdot 2H_2O$, 416; $MgCO_3$, 25; $MgSO_4 \cdot 7H_2O$, 30; NaCl, 127; KCl, 21; $FePO_4 \cdot 4H_2O$, 20; KH_2PO_4, 315; $MnSO_4 \cdot 4H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 1.6; $Al_2(SO_4) \cdot K_2SO_4 \cdot 24H_2O$, 0.24; KI, 0.6; $CoCl_2 \cdot 6H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.05; and NaF, 1.0.

Diet B (43% protein) was prepared in the same manner by the addition of 36.7 gm of casein (alcohol extracted), 10.5 gm of gelatin, and 24.3 gm of starch.

Diet C (25% protein) was an open formula, commercial starting mash of the following percentage composition: wheat bran, 8; wheat standard middlings, 7; crushed wheat, 11; yellow corn meal, 15.5; low fiber oats, 7; alfalfa meal, 7.5; soybean oil meal (41%), 24; meat scrap (55% protein), 4; fish meal, 4; brewers' yeast, 3; riboflavin supplement, 0.4; dried whey, 5; ground limestone, 2; dicalcium phosphate, 1; iodized salt, 0.5; and "D" activated animal sterol, 0.1. Manganese sulfate was included in the ration so that each 100 gm contained 0.018 gm. Dried whey was fortified with riboflavin to contain a minimum of 5 mg per 100 gm. The D-activated animal sterol provided 200 A.O.A.C. chick units of vitamin D per 100 gm of ration.

Synthetic folic acid was added as an alcoholic, buffered solution containing 0.5 mg per ml, at the feeding levels shown in the tables.

RESULTS AND DISCUSSION

Growth

After 40 days (48 days of age) and after 56 days (64 days of age) on experiment, the poults receiving 43% protein, diet B, were significantly heavier than those on diet A, containing 25% protein, at all levels of folic acid supplementation (table 1). For each protein level, the weights at 40 days showed a tendency to be greater as the quantity of folic acid in the ration was increased, except that groups 14 and 17 showed lower body weights than those of the groups receiving the next lowest level of folic acid. At this time the poults fed the starting mash, group 18, weighed less even than those on diet B with 0.5 mg of folic acid.

After 56 days on experiment, too few birds remained in groups 10, 11 and 13 to permit their inclusion in a comparison of body weights. Those consuming diet B and 1.5 and 2.0 mg of folic acid per kg of feed were significantly heavier than

those on the corresponding levels of folic acid and being fed diet A (25% protein). This difference was undoubtedly due to the difference in protein levels. At this time, the poults fed diet C (starting mash) were slightly but not significantly heavier than the group on diet B and a 2.0 mg level of folic acid. Actually, only 2 poults on the starting mash were heavier than those receiving diet B.

TABLE 1

Growth of turkey poults on graded levels of folic acid.

GROUP NO.	FOLIC ACID	INITIAL WEIGHT 8 DAYS	WEIGHT, 40 DAYS OF EXPERIMENT; 48 DAYS OF AGE	WEIGHT, 56 DAYS OF EXPERIMENT; 64 DAYS OF AGE
	mg/kg	*gm*	*gm*	*gm*
		Diet A. 25% protein		
8	0.0	68 (6)[1]	... (0)	... (0)
10	0.5	74 (10)	418 (8)	(490)[2] (1)
12	1.0	74 (10)	449 (10)	608 (10)
14	1.5	72 (10)	422 (10)	643 (10)
16	2.0	74 (10)	469 (10)	682 (10)
		Diet B. 43% protein		
9	0.0	71 (6)	... (0)	... (0)
11	0.5	74 (10)	579 (6)	(600) (1)
13	1.0	71 (10)	659 (7)	(648) (4)
15	1.5	72 (10)	725 (8)	918 (8)
17	2.0	73 (10)	684 (8)	974 (7)
		Diet C. 25% protein in starting mash		
18	Starting mash	74 (10)	562 (9)	1017 (9)

[1] Poults per group.

[2] Insufficient number of poults for comparison.

The greater gains noted, after 40 days on the experimental diet in the case of all of the groups fed 43% protein and folic acid supplements, in contrast with the gain on the starting mash, may have been due to the fact that the pattern and quantity of amino acids presented by the casein and gelatin of diet B met more adequately the requirements for early rapid growth than did the ingredients of diet C, the starting mash. At 56 days these requirements may have been less,

or the folic acid supplements, except for the 2.0 mg level, may have been inadequate for the heavier birds.

Cervical paralysis

Observations of the development and incidence of cervical paralysis are shown in table 2. Paralysis occurred in all groups

TABLE 2

Development of cervical paralysis and mortality in turkey poults.

GROUP NO.	INITIAL NO. OF BIRDS	FOLIC ACID	AVERAGE NO. DAYS TO PARALYSIS	DAYS TO FIRST CASE	DAYS TO LAST CASE	AVERAGE NO. DAYS TO DEATH	MORTALITY	
							Cervical paralysis no.	Other causes
		mg/kg						
				Diet A. 25% protein				
8	6	0.0	37	36	37	38	5	1 nonpathological
10	10	0.5	44	33	56	45	9	1 nonpathological
12	10	1.0	62	59	64	63	7	
14	10	1.5	59	59	59	62	2	1 nonpathological 1 perosis
16	10	2.0	0	0	0	0	0	2 nonpathological
				Diet B. 43% protein				
9	6	0.0	27	21	30	29	6	
11	10	0.5	41	32	56	43	10	
13	10	1.0	50	34	55	51	5	1 nonpathological 2 perosis
15	10	1.5	52	54	68	53	4	2 nonpathological 1 perosis
17	10	2.0	0	0	0	0	0	2 nonpathological
				Diet C. 25% protein in starting mash				
18	10	Starting mash	0	0	0	0	0	1 nonpathological

on the purified diet at both protein levels, except in those which received 2.0 mg of folic acid, the incidence decreasing with an increase in the folic acid level.

On diet A (25% protein), without folic acid, 5 of the 6 poults in the group died following the onset of cervical paralysis and the other member died of a nonpathological cause 2 days

after the start of the experiment. In this group the first case of paralysis was noted 36 days after the start of the experiment and all 5 poults that developed paralysis were dead by the thirty-eighth day. Among the poults of group 9, consuming diet B (43% protein) without a folic acid supplement, the first case appeared after 21 days of experiment and the last after 30 days, the average number of days to death being 29. The 6 poults of this group all died of cervical paralysis. It is noteworthy that on the 43% protein level, paralysis appeared 15 days earlier and the average number of days until death was 9 less than that for the group consuming diet A (25% protein). This difference in the onset of paralysis may be due to the more rapid growth at the higher protein level.

On both protein levels, when the folic acid supplied was 0.5 mg, all of the birds died of cervical paralysis except 1 on diet A which died of a non-pathological cause 12 days before the first case of paralysis occurred. In these groups and in those receiving 1.0 and 1.5 mg of folic acid paralysis appeared earlier and the average number of days to death was less at the 43% protein level than at the 25% level.

Although at the 1.5 mg level of folic acid, 2 cases of cervical paralysis appeared in the group which received diet A (25% protein) and 4 in the group fed diet B (43% protein), an increase of the folic acid supplement by 0.5 mg per kg of feed, so that the supplement was 2.0 mg, gave complete protection against the development of paralysis. No cases of paralysis appeared on diet C, the starting mash.

Feathering

Throughout the experiment the feathering of all poults, except those receiving 2.0 mg of folic acid and diet B (43% protein), was subnormal as compared with that of the poults on the starting mash. The feathering departed from the normal in that breast feathers did not fit the body, and the tail feathers were sparse and broken. The wing feathers were

ragged and broken and did not lie close to the body. The feathering of poults on diet B (43% protein) and 2.0 mg of folic acid was indistinguishable from that of poults fed the starting mash, except that a few individuals showed some broken feathers which may have been due to cage damage and a tendency for the wings to droop. The light tan color of the Jersey buff poults was true to type for all groups

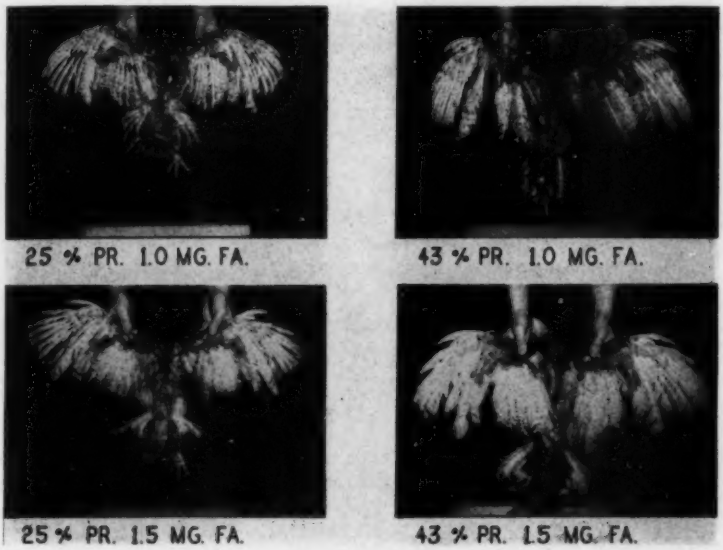

Fig. 1 Feathering of poults in relation to protein (PR.) and folic acid (FA.) levels, per kg of ration.

except those which received 43% protein and 2.0 mg of folic acid, in which case the color was definitely darker. The darker brown color of the plumage was also noted to an even more marked degree in an earlier experiment.

Wing and tail feathers of typical subjects after 56 days on experiment are shown in figures 1 and 2. Using the feathering of the poults fed the starting mash as normal, it is apparent that only for the poults on the purified diet B, which

provided 43% protein and 2.0 mg of folic acid, was the wing and tail feathering the same as that of the poults considered to be normal.

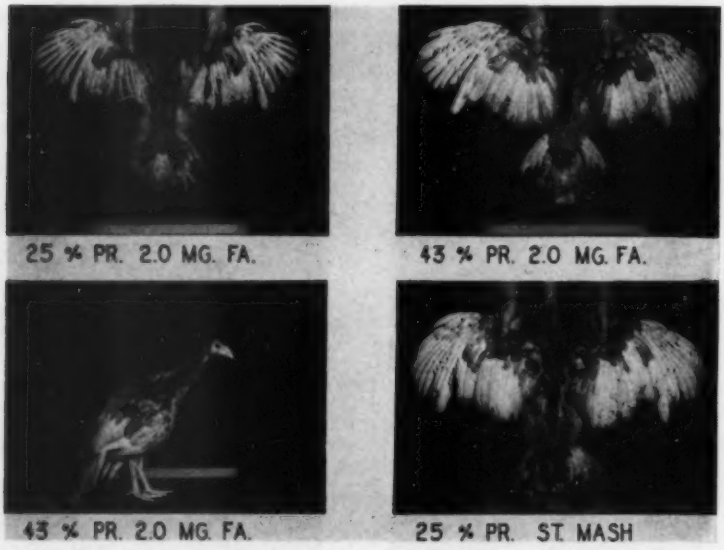

Fig. 2 Feathering of poults in relation to protein (PR.) and folic acid (FA.) levels, per kg of ration and a commercial turkey starting mash (ST.MASH).

Hematology

Hemoglobin values were obtained from samples drawn from the heart at intervals and as soon as signs of cervical paralysis appeared, the method of Schultze and Elvehjem ('34) being used. Readings were made with a Pfaltz and Bauer photoelectric colorimeter and values per gram of hemoglobin per 100 ml blood were obtained from a standard curve for which dilutions of an acid hematin standard solution were used. The readings were all taken with the diaphragm at 10 and using a 5200–5300 Å filter. In table 3 are listed the group averages of the hemoglobin determinations. The group receiving

the turkey starting mash has been taken as normal in considering the relative hemoglobin values.

It is evident that the hemoglobin values of the blood do not increase in direct proportion to the folic acid content of the feed. For none of the poults which survived 72 days of experiment on the purified ration at the 25% protein level, was the hemoglobin value as high as that obtained, at the corresponding folic acid level, when 43% protein was fed, indicating that the quantity of protein in the ration influences the hemoglobin content of the blood. On the purified ration containing 43% protein, only the group which received 2.0 mg

TABLE 3

Hemoglobin values for turkey poults at various levels of folic acid supplementation.

GROUP NO.	FOLIC ACID	PROTEIN	AVERAGE Hb OF POULTS WHICH DEVELOPED PARALYSIS	AVERAGE Hb OF SURVIVORS (72 DAYS)
	mg/kg	%	*gm/100 ml*	*gm/100 ml*
8	0.0	25	6.7	..
10	0.5	25	6.6	..
12	1.0	25	7.3	9.8
14	1.5	25	6.1	8.8
16	2.0	25	..	10.5
9	0.0	43	6.9	..
11	0.5	43	5.7	..
13	1.0	43	6.4	12.3
15	1.5	43	6.2	11.6
17	2.0	43	..	12.7
18	Starting mash	25	..	13.0

of folic acid had a hemoglobin value essentially of the same order as that shown by the poults on the starting mash. The values for the blood of the paralytics, however, are essentially the same at both protein levels except that some lower values occurred in the groups on 43% protein. Jukes et al. ('47), on the other hand, have reported a hemoglobin level of 10 gm at 4 weeks on a diet containing approximately 25% protein.

General appearance

Frequent examination of the eyes, the skin of the feet, and the head did not reveal any abnormalities. Except for 4 cases

of perosis the posture of the birds was normal. All of the birds on the purified diet had pendulous crops in an earlier experiment, but only a few cases were observed in the present trial.

GENERAL DISCUSSION

It is evident that, of the 2 percentages of protein used in the purified diets, 25% and 43%, the higher at each folic acid level promoted better growth and feather formation and higher hemoglobin. Luckey and coworkers ('46) have concluded that for the chick a definite requirement for folic acid cannot be established because the response to a given quantity depends upon the type of ration used. The diet used by these investigators, which approached most closely the composition of purified diet A (25% protein) of the present experiment, promoted at 4 weeks of age, when the folic acid level was 1.0 mg per kilo, growth and hemoglobin formation inferior to that noted when corn meal replaced starch and contributed part of the protein, and 0.5 mg of folic acid was supplied per kilogram. The corn meal diet used by these investigators is the closest approach to the starting mash used in our experiment. These workers reported normal feathering in the chick when a purified type of diet was fed containing approximately 25% protein (by calculation) from casein and gelatin, and 0.5 mg of folic acid per kilogram of diet. When the protein of the diet, approximately 40% (by calculation) was supplied by casein only, subnormal feathering was obtained even when 3.0 mg of folic acid per kilogram of diet was provided. These observations and the difference in the feathering of poults at 2 protein levels in the present experiment suggest that, as long as other requirements are met by known factors, it may be the quantity of amino acids essential for feathering, that is responsible for normal feathering rather than a specific feathering factor.

Robertson et al. ('46) report that for the chick, at 6 weeks of age, approximately 0.25 mg of folic acid per kilogram of diet is necessary for survival; 0.45 mg for growth; 0.35 mg for

hemoglobin formation and not less than 0.55 mg for feathering. The diet used by these investigators was of the purified type, containing 30% protein (by calculation) plus a factor S concentrate equivalent to 5% yeast. The turkey poult, according to the results reported in the present paper, requires at least 4 times the quantity of folic acid for the several functions listed as does the chick. The higher requirement is in agreement with the generally accepted observation that the turkey poult has a greater need for certain nutrients as compared with the chick.

SUMMARY AND CONCLUSIONS

The folic acid requirement for normal growth of poults on a purified ration is 2.0 mg per kilogram of feed.

At the 25% and 43% protein levels of the purified ration, 2.0 mg of folic acid per kilogram of feed is required to prevent cervical paralysis. No cervical paralysis occurred in the group which consumed the turkey starting mash.

When the level of folic acid was 2.0 mg per kilogram of feed, normal feathering was observed in the poults receiving the purified diet with 43% protein, but at the same folic acid level those fed the purified diet containing 25% protein showed feathering that was distinctly subnormal.

Although the hemoglobin values for the poults which did not develop cervical paralysis showed no direct relationship to the level of the folic acid supplement on either of the purified diets, they were higher, 11.6–12.7 gm, on the diet containing 43% protein than those on the diet containing 25% protein, 8.8–10.5 gm. Those obtained on the 43% protein diet were essentially of the same order as that observed with the poults on the turkey starting mash. For the birds which developed cervical paralysis, the hemoglobin values were lower and ranged from 5.7 to 7.3 gm per 100 ml of blood.

ADDENDUM

Since this manuscript was completed, Lillie and Briggs [Poultry Sci., *26:* 295 (1947)] have reported that New Hamp-

shire chicks require a minimum of 150 μg of folic acid per 2.0 mg of folic caid per kilogram of feed is required to prevent and that 200 μg per 100 gm of diet (2.0 mg per kilo) is the requirement for normal feathering.

ACKNOWLEDGMENTS

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THE NUTRITIVE VALUE OF CANNED FOODS[1]

XXV. VITAMIN CONTENT OF CANNED FISH PRODUCTS

J. B. NEILANDS, F. M. STRONG AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

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Although a large number of canned meats and vegetable products have previously been assayed for vitamin content the number of fish products which have been thus investigated is relatively small. Recently it has been reported (Deuel et al., '45) that at least some fish proteins are very complete with respect to the growth requirements of the rat. The following investigation was undertaken to determine the quantitative distribution of certain growth factors in different species of canned fish.

EXPERIMENTAL

Samples

The method of collection of the samples has previously been outlined by Clifcorn ('44). Each sample consisted of 72 cans of the regular retail product and in most instances a report bearing a description of the location and date of catch, canning procedure, etc., accompanied each sample.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The vitamin D assays reported in this paper were carried out at the Wisconsin Alumni Research Foundation laboratories under the direction of Dr. H. T. Scott, whose assistance is gratefully acknowledged. Thanks are due also to Mr. A. L. Powell, Jr., who did most of the thiamine determinations.

This work was a part of the National Canners Association-Can Manufacturer's Institute Nutrition Program.

Twelve cans were opened, the contents combined, and the solid and liquid portions separated with a fine screen. The liquid fractions were discarded except in the case of several of the products canned in vegetable oil. A few of these oils were analyzed for their vitamin A and D contents. The solids were then mixed by hand as thoroughly as possible, passed through a commercial meat grinder, mixed again and stored in the dark at a sub-zero temperature. All assays were carried out before this preparation was over 6 weeks old. For assays of vitamins A and D this material was used directly or if the oil was assayed it was simply clarified by filtration.

The water-soluble vitamin assays were carried out on a second preparation made from time to time by mixing an equal weight of the above material and distilled water in a Waring Blendor. All assays, except those for folic acid and vitamin D, were done in duplicate. The samples packed in tomato sauce were not separated into the solid and liquid fractions but the entire contents of the cans were prepared and assayed in the same manner as the solid portion referred to above.

The 11 samples of salmon assayed consisted of 5 different species of the genus *Oncorhynchus*, representing what are commonly called red, chinook, chum, coho and pink salmon.

Assay methods

Vitamin A. Vitamin A was determined by the antimony trichloride method of Dann and Evelyn ('38) using the saponification procedure of Jensen and With ('39). It was found that the vitamin A content of the muscle tissue and oil was extremely low. In addition considerable carotenoid pigment appeared in the final chloroform extract although much of this could be removed by the separation procedure of Boyer, Phillips and Smith ('44). This separation procedure was used for the analyses of the oil from the Atlantic sardines and mackerel. The blue color of the vitamin A-antimony trichloride complex was much more stable in extracts made by

saponification of the oil than by saponification of the muscle of these 2 species.

Most species of salmon examined contained varying amounts of the astacin-like pigment described by Bailey ('37). In making saponifications of the whole muscle it was found that this pigment formed an alkali salt slowly and only after refluxing for a period of 2 hours was it almost completely removed with the saponified fraction. This technique apparently permitted fairly accurate checks to be obtained in the salmon analyses.

Thiamine. The thiochrome method of Hennessy ('42) as outlined in the U.S. Pharmacopoeia ('47) was used for thiamine analyses. After incubation of the samples with the proteolytic and diastatic enzyme ("polidase")[2] the digests were extracted with ether to facilitate their passage through the adsorption columns.

Riboflavin. For riboflavin the microbiological method recommended by Strong ('47) was used. Since all samples contained appreciable quantities of fat the techniques suggested for its removal were employed.

Pantothenic acid. The *L. casei* method of Neal and Strong ('43) was used for pantothenic acid. The vitamin was liberated by digestion with "mylase P" according to Ives and Strong ('46). Precautions to remove fat were again exercised.

Biotin. L. arabinosus was used to measure biotin (Wright, '47).

Nicotinic acid. L. arabinosus was also used to assay nicotinic acid. The technique used was that of Snell ('47).

Pyridoxine. The *S. carlsbergensis* method of Atkin et al. ('43) was used with certain modifications of incubation procedure for the determination of pyridoxine activity. Either shaking in large test tubes (2.2 × 20 cm) or stagnant incubation in 125-ml Erlenmeyer flasks proved capable of providing the desired turbidity in 18–20 hours at 30°C.

Folic acid. In each case the samples for folic acid were treated with a crude conjugase preparation. The latter was

[2] Recommended by Clausen and Brown ('43).

TABLE 1

The vitamin content of the solid portion of canned fish.

SPECIES		ATLANTIC MACKEREL [1] (*Scomber scombrus*)	ATLANTIC SARDINES [1] (*Clupea harengus*)	TUNA (*Germo, Neothunnus, Thunnus* and *Katsuwonus*)	SALMON (*Oncorhynchus*)	PACIFIC MACKEREL (*Pneumatophorus* and *Trachurus*)	PACIFIC SARDINES (*Sardinops caerulea*) Tomato sauce	PACIFIC SARDINES (*Sardinops caerulea*) Natural
NO. OF SAMPLES		4	8	12	11	5	5	6
FACTOR		mg per 100 gm						
Vitamin A	Range	0.065–0.274	0.043–0.118		0.017–0.097			
	Ave.	0.130	0.067	<0.008	0.036	<0.008	<0.008	<0.008
Thiamine	Range	0.040–0.086	0.003–0.028	0.040–0.066	0.018–0.052	0.018–0.038	0.006–0.017	0.000–0.019
	Ave.	0.058	0.015	0.053	0.030	0.026	0.010	0.007
Riboflavin	Range	0.17–0.26	0.16–0.23	0.09–0.15	0.11–0.20	0.29–0.38	0.29–0.38	0.23–0.37
	Ave.	0.20	0.19	0.11	0.16	0.33	0.33	0.30
Pantothenic acid	Range	0.25–0.40	0.42–0.54	0.35–0.56	0.47–0.69	0.43–0.54	0.39–0.49	0.49–0.83
	Ave.	0.31	0.47	0.42	0.58	0.47	0.45	0.60
Biotin	Range	0.003–0.004	0.003–0.006	0.003–0.006	0.011–0.019	0.012–0.023	0.020–0.033	0.019–0.034
	Ave.	0.003	0.004	0.003	0.015	0.018	0.027	0.024
Nicotinic acid	Range	5.0–6.6	3.8–5.9	10.2–15.5	6.2–8.1	8.0–9.4	5.4–6.4	6.2–8.8
	Ave.	5.8	4.8	13.5	7.4	8.7	5.8	7.4
Pyridoxine	Range	0.18–0.26	0.13–0.20	0.37–0.52	0.39–0.50	0.25–0.29	0.19–0.25	0.24–0.30
	Ave.	0.21	0.16	0.44	0.45	0.27	0.22	0.28
Folic acid	Range	0.0009–0.0022	0.0008–0.0027	0.0006 [2]	0.0005 [2]	0.0006 [2]	0.0008 [2]	0.0005 [2]
	Ave.	0.0013	0.0016					

[1] Vitamin A analyses on oil drained from the can contents.

[2] One sample only assayed.

prepared by disintegrating in a Waring Blendor, 1 part of fresh hog pancreas with 3 parts of water (weight to weight) and filtering to remove the large particles. This conjugase treatment usually increased the level of the vitamin from 50 to 70 fold as measured by the *L. casei* method of Teply and Elvehjem ('45).

TABLE 2

Vitamin D content of canned fish.

SAMPLE [1]	USP VIT D UNITS PER 100 GM	SAMPLE [1]	USP VIT D UNITS PER 100 GM
Atlantic mackerel		Salmon	
1	250	1	500
2	250+	2	500
3	165	3	500+
4	250	4	500
Pacific sardines		5	500
1	333	6	500
2	333	7	500
3	333	8	500+
4	333	9	500
5	333	10	500
6	333	11	500
Atlantic sardines		Pacific sardines with tomato sauce	
1	250	1	333
2	333	2	333
3	250+	3	333
4	250	4	333
5	250	5	500
6	250+	Pacific mackerel	
7	333	1	250
8	250	2	250
Tuna		3	250
1	200	4	250
2	200	5	250
3	250	Atlantic sardine oil [2]	333
4	<100	Pacific sardine oil [2]	333
5	250	Tuna oil [2]	
6	225	3	333+
7	250	6	333
8	250	8	<100
9	250	12	333+
10	250		
11	250+		
12	333		

[1] Samples are the same as in table 1.

[2] Oils drained from the can contents.

Vitamin D. Rat assays for vitamin D were carried out according to the U.S.P. method. The amounts fed were such as to permit estimation of the potency with a precision indicated by the levels given in table 2, *e.g.*, 165, 200, 250, 333, 500 U.S.P. units per 100 gm. Closer estimation of the potency was not considered feasible on account of the excessive number of individual assay levels required.

DISCUSSION

Unfortunately very few analysts report the scientific name of the fish being sampled. This leads to some confusion as to whether, for example, a particular species is of Atlantic or Pacific origin and renders comparison with literature results rather difficult.

Vitamin A. Considerable variation was found in the vitamin A analyses of the different species of canned salmon. Thus 2 samples of red, 1 sample of sockeye (red) and 1 of chinook salmon contained 0.045, 0.045, 0.095 and 0.070 mg of vitamin A per 100 gm, respectively, whereas 3 samples of pink, 2 samples of coho and 2 of chum salmon ranged from 0.017 to 0.026 mg of vitamin A per 100 gm. No comparable variation in the B vitamin content of the salmon samples was observed.

Recently Awapara et al. ('46) have described a new spectrophotometric method for the determination of vitamin A. It is possible that this method could be applied to food samples where vitamin A is in small concentrations although it was unsatisfactory for measuring the vitamin in rat livers.

Since all samples contained some carotenoid pigment it was possible that higher values for vitamin A activity could be found by the rat assay. Devaney and Putney ('35) found 0.25 to 8.0 I.U. per gm of vitamin A in the flesh of several species of canned salmon. This range was approximately the same as reported in the present paper. The flesh of the other species assayed in the present work contained less than 0.008 mg of vitamin A per 100 gm. Until additional animal assay data are available it will

not be possible to draw final conclusions as to the value of canned fish as sources of vitamin A.

Thiamine. Guerrant, Vavich and Fardig ('45) have reported a survey carried out in a number of different laboratories. The thiamine content of a salmon purée varied between 0.0084 and 0.0104 mg per 100 gm when assayed by the thiochrome method. Some of the lower values reported in the present survey (table 1) may be due in part to thiaminase action in the muscle prior to canning. Thus Melnick et al. ('45) have found that herring contains this enzyme as does also California sardines (Lamb, '46). Myrback and Vallin ('44) have found that the canning process destroys 30% of the original thiamine in canned meat. These factors tend to decrease the value of canned fish products as a source of thiamine.

Riboflavin. The riboflavin content of the various samples within a given species showed remarkably little variation. In the Pacific sardines and mackerel the values were somewhat higher and can be regarded as comparable to lean beef or pork.

Pantothenic acid. All of the canned fish samples analyzed proved to contain approximately the same levels of pantothenic acid as those found in other muscle meats, namely, 0.25 to 0.83 mg per 100 gm for the fish as compared, for example, to 0.45 for pork (Guerrant, Vavich and Fardig, '45).

Very recently Lipmann et al. ('47) have reported that pantothenic acid may be the coenzyme for an acetylating enzyme for choline, sulfanilamide, etc. This discovery will add impetus to the search for more quantitative data on the distribution of this factor in foods.

However, the most serious problem in the estimation of pantothenic acid is its liberation from other materials in the sample. Incubation of the samples with "mylase P" was previously found to release more of the vitamin than treatment with other common proteolytic enzymes (Ives and Strong, '46); hence this technique has been applied in the present investigation. It is interesting to note that Lipmann

has found an enzyme in liver which together with a phosphatase liberated from the purified acetylating coenzyme 60 times as much pantothenic acid as could be obtained by clarase-papain digestion.

Biotin. Ives et al. ('46) found an average of 0.0098 mg of biotin per 100 gm in 10 samples of salmon assayed by the *L. casei* method of Shull and Peterson ('43). In the present study the Pacific sardines proved to be a somewhat better source of biotin than the other species.

There are several reports in the literature on the biotin activity of certain fatty materials such as, for example, oleic acid. It is possible that this factor may have been at least partially responsible for the fluctuations noted although all samples were filtered at a pH of 4.0–4.5 in order to remove fatty acids.

Nicotinic acid. All species can be stated to be a good source of nicotinic acid. The values found for the canned tuna samples were much higher than those usually reported in the literature for beef and ham.

Pyridoxine. The average pyridoxine value for the 10 salmon samples assayed by Ives et al. ('45) was 0.13 mg per 100 gm. The distribution of this vitamin in tuna and salmon, reported in table 1, is somewhat higher than most literature values for meat.

Folic acid. The assays reported for folic acid show that the various fish products are very low in this vitamin. The method of Roberts and Snell ('46) using *L. casei* as the test organism did not give more satisfactory results. There is some indication that this factor may be destroyed in the cooking of meat (Schweigert, Pollard and Elvehjem, '46), hence it is possible that the canning process may also destroy this factor.

Although the samples were treated with a conjugase preparation, it may be possible that not all the folic acid was actually liberated.

Vitamin D. The rather high levels of vitamin D found for the various species is one of the most interesting results of the present survey. From the values reported in table 2, it

may be seen that these species of fish are excellent sources of vitamin D and as such are unique among commonly eaten, unfortified foods. In view of the practical importance of this fact, the vitamin D values are given in full in table 2.

Devaney and Putney ('35) assayed 4 species of canned salmon and found the vitamin D content to range from 190 to 800 I.U. per 100 gm. The average figure taken from the results of the above authors is 460 I.U. per 100 gm, about the same value as that found in the present work (table 2).

It may be concluded that, with the exception of folic acid, the B vitamin content of the canned fish products assayed in the present study compares favorably with that of other major protein foods such as muscle meats, eggs, and cheese. In addition canned fish may be considered an excellent source of vitamin D, and should probably be regarded a rather poor source of vitamin A.

SUMMARY

A number of samples of canned fish representing 6 different species were tested for their content of vitamins A, D and certain water-soluble vitamins.

No appreciable amount of vitamin A was found in the flesh of any of the canned fish assayed except salmon, although small amounts were contained in the oils from canned Atlantic mackerel and sardines. Thiamine and folic acid were generally rather low. Riboflavin, pantothenic acid and biotin were present in fair amounts. The samples proved to be a good source of nicotinic acid and pyridoxine. All species examined were good sources of vitamin D.

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THE INFLUENCE OF PTEROYLGLUTAMIC ACID (A MEMBER OF THE VITAMIN M GROUP) ON GASTROINTESTINAL DEFECTS IN SPRUE. A STUDY OF INTERRELATIONSHIPS OF DIETARY ESSENTIALS[1]

WILLIAM J. DARBY, EDGAR JONES, HENRY F. WARDEN AND MARGARET M. KASER

Departments of Medicine and Biochemistry, Vanderbilt University School of Medicine, Nashville; and Tennessee Department of Public Health, Nashville

FIVE FIGURES

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Patients with the complete syndrome of sprue may exhibit the most remarkable combinations of deficiency states (Thaysen, '32; Hanes, '46; Wintrobe, '46). Some of the manifestations of these states are loss of weight (caloric deficiency), hypoproteinemia (protein deficiency), petechiae (vitamin K or C deficiency) (Fanconi, '38; Kark, Souter and Hayward, '40; Butt and Snell, '41), night blindness and xerosis conjunctivae (vitamin A deficiency) (Riddell, '33; Rao, '33), hypocalcemic tetany and osteoporosis (vitamin D and calcium deficiency) (Scott, '25; Linder and Harris, '30; Fairley, '36), and pancytopenia (vitamin M group deficiency)[2]

[1] This work was supported by grants from the National Vitamin Foundation, the Nutrition Foundation, Inc., the International Health Division of The Rockefeller Foundation, and Distillation Products, Inc.

[2] The term "deficiency" is employed here in its broad sense. We do not wish to imply that the deficiency is necessarily dietary. It is conceivable that some cases may be dietary in origin, but it is probable that some constitutional conditioning factor and/or environmental conditioning factor exerts a major influence upon the development of the disease-syndrome termed sprue.

(Darby and Jones, '45). In many cases these deficiencies may be classified (Dann and Darby, '45) as latent or potential and, hence, are demonstrable only by biochemical means.

The view that sprue is a primary disease was clearly argued by Thaysen ('32). Hanes ('43) held that the " . . . . profound disturbance of the power of the intestines to absorb in a normal fashion . . . " would explain all of the varied secondary deficiency manifestations of the syndrome. The association of sprue with dietary inadequacies had often been remarked upon (Ashford, '22; Thaysen, '32), but Castle and associates ('35) clearly hypothesized that "In sprue the obvious dietary defects provide . . . a rational explanation of the subsequent defects of the specific functions of the alimentary tract demonstrated in patients." These gastrointestinal defects are at least partially corrected by a substance or substances present in liver extract (Castle et al., '35; Fairley, '36; Miller and Rhoads, '36; Barker and Rhoads, '37; Miller and Barker, '37).

With the demonstration of the activity of pteroylglutamic acid (PGA) in patients with sprue, we (Darby and Jones, '45; Darby, Jones and Johnson, '46a, b; Darby, Jones, Warden and Kaser, '47) indicated that this vitamin seemed to exert a favorable influence upon the gastrointestinal abnormalities as well as upon the anemia. Similar observations have been made by others (Beguerie and Spies, '46; Suárez, Spies and Suárez, '47). This paper summarizes additional data which may be interpreted to indicate that pteroylglutamic acid exerts a favorable influence upon the gastrointestinal tract in sprue.

EXPERIMENTAL

The subjects were 6 adult patients with sprue who have been observed in the Vanderbilt University Hospital. All of these cases met the diagnostic criteria of glossitis, diarrhea with an increased fat content of the stools, loss of weight, macrocytic anemia, moderate leucopenia and definite impairment of gastrointestinal absorption as will be discussed. Some of the initial data on 4 of these cases have been previously

reported (Darby, Jones and Johnson, '46a; Jones, Warden and Darby, '47). One patient (J.D.), however, has now been observed during a second relapse after the withdrawal of therapy and through a second remission induced by PGA. This makes a total of 7 observed remissions reported herein; each remission is considered independently. The sole specific therapeutic agent administered has been synthetic pteroylglutamic acid [3] in doses of either 5 mg per day orally or 15 mg per day intramuscularly, except in 1 case (Jones, Warden and Darby, '47) in which this was preceded by 10 days of therapy with pteroyltriglutamate. With 1 exception (J.H.), the patients have been hospitalized during the acute stage of their disease. The prehospital dietaries of the patients varied considerably. They had been generally of poor quality, but not uniformly so. During therapy 1 patient (P.B.) received a meat-free diet; the remainder have been maintained on a regular ward diet devoid of organ meats. No dietary advice whatsoever has been offered the patients upon their return home. Other than indicated in figure 2a no other supplementary vitamins have been administered.

Glossitis

During each relapse the patient has complained of a sore tongue, particularly sensitive to acid foods, spices and the like. All of the patients have shown atrophic lingual papillae. Subjective relief of the glossitis has occurred and beginning regeneration of the papillae has been evident within 3 days to a week after the institution of therapy. In 6 of the 7 remissions induced by PGA there has been complete restoration to normal of the lingual changes. The other case (J.H.) has shown pronounced improvement. These successive lingual changes observed in J.D. prior to therapy, following administration of PGA, in a relapse after the interdiction of therapy,

[3] We are indebted to Drs. Stanton M. Hardy, Thomas H. Jukes, and E. L. R. Stokstad of the Lederle Laboratories for their generous assistance in supplying us with synthetic pteroylglutamic acid and the isolated pteroyltriglutamate employed in this study.

and in a second remission induced by PGA are shown in figure 1. If, as is sometimes held, the appearance of the tongue in patients with sprue is an indicator of the state of the gastrointestinal mucosa generally, these findings would

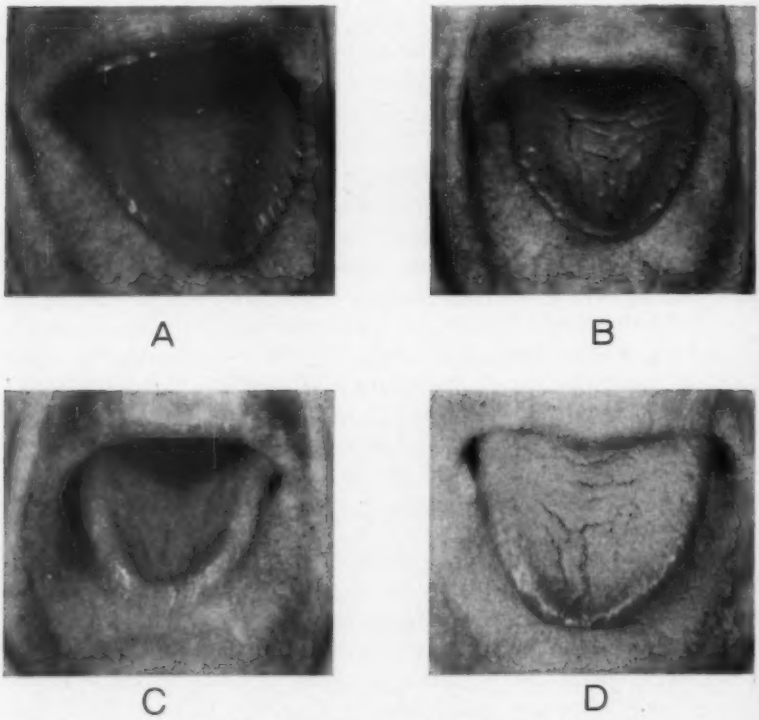

Fig. 1 a. Tongue of patient J.D. on September 26, 1945, prior to therapy. Note the complete absence of lingual papillae.

b. Tongue of patient J.D. photographed on October 18, 1945. Therapy with PGA was instituted on October 4, 1945. Note the evident regeneration of papillae which impart a velvety appearance to the central area.

c. Tongue of patient J.D. photographed on July 24, 1946, 275 days after the last injection of PGA. The patient was in a clinical relapse. Note the similarity in appearance to the photograph made on September 26, 1945.

d. Tongue of patient J.D. photographed on December 4, 1946, at the height of the second remission induced by PGA. Complete regeneration of the lingual papillae is evident.

indicate a definitely beneficial effect of this vitamin on the mucosa.

Diarrhea

In all of these remissions cessation of diarrhea has been observed within a period of 3 to 9 days from the beginning of therapy with PGA. In 1 case (J.H.) periodic recurrence of this symptom has taken place, but the frequency of recurrence has decreased. As noted below, this patient has developed niacin deficiency while receiving PGA.

Weight changes

The maximum weight gains in pounds have been as follows: 45, 26 (J.D.); 33 (P.S.); 24 (P.B.); 29 (W.O.); 6 (J.H.). It is our impression that these weight changes following therapy reflect both the increased appetite and the better absorptive capacity of the gastrointestinal tract with the consequent conservation of calories and fluid. Several of our patients have exhibited evidence of fluid retention during the early period of therapy (Darby, Jones and Johnson, '46a).

Glucose tolerance

It has been possible to determine the glucose tolerance (oral administration) in 5 of these remissions. In each instance there has been a definite return toward the normal type of curve within from 11 to 30 days after institution of therapy. These changes are illustrated by figures 2a and 2b. Other data on the glucose tolerance were presented elsewhere (Darby, Jones and Johnson, '46a). The dosage of glucose employed in these studies has been 70 gm routinely. Confirmatory findings have been reported by Suárez et al. ('47). Crawford ('39) has interpreted the low glucose tolerance curves as indicative of defective or delayed absorption of carbohydrate from the intestine. Fairley ('36) regards the transition from the flat type of glucose tolerance curve to the normal type following therapy with liver extract as indicating improved absorption of carbohydrate. By similar

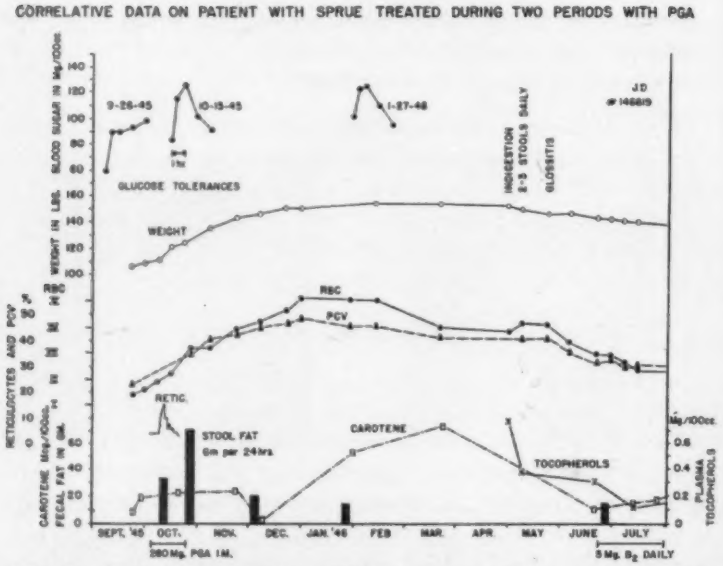

Fig. 2a See text for discussion. Riboflavin, 5 mg daily by mouth, was given as a placebo from June 26 to July 14, 1946. The course of the relapse was unaltered by this therapy.

reasoning, one may hypothesize that PGA favorably influences the permeability of the gastrointestinal tract to glucose.

Fat soluble factors

It is essential that the dietary intake of fat be properly controlled (Black, Fourman and Trinder, '46) in order that quantitative analyses of stool fat be interpreted with accuracy. This control of the fat intake of patients is extremely difficult, especially after the subject returns home. Our observations on the effect of PGA on fecal fat are, therefore, exceedingly limited, but successive determinations on a single patient (figs. 2a and 2b) indicate a definite decrease in the total 24-hour loss of fat in the stool. In chronic sprue, however, PGA has not reduced the loss of fat in the stool beyond that point attained by previous liver extract therapy

(Davidson, Girdwood and Innes, '47). Spies et al. ('46) have observed that diarrhea was better controlled by PGA in patients whose illness was of recent onset.

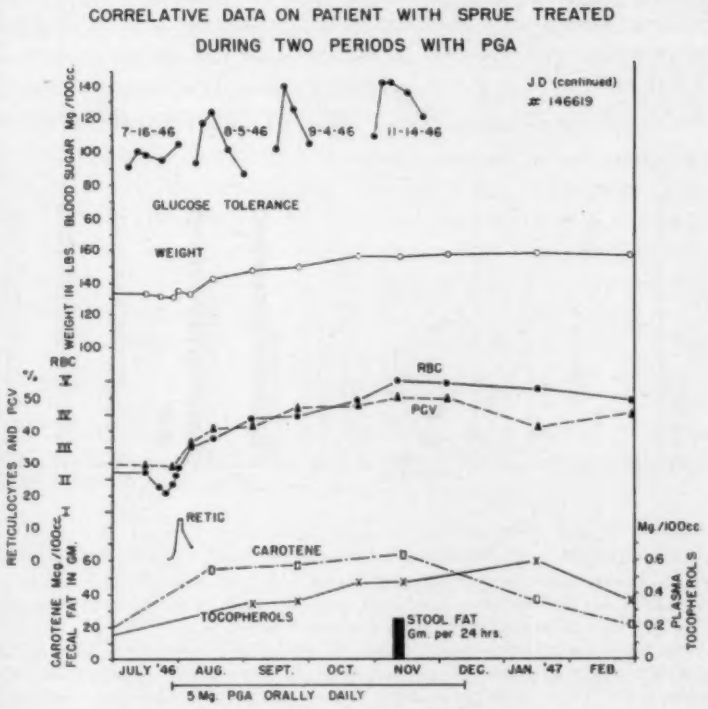

Fig. 2b See text for discussion.

Carotene and vitamin A

Some data on the absorption of carotene and vitamin A have been presented in a previous communication (Darby, Kaser and Jones, '47). The hematologic pictures of sprue and pernicious anemia are identical and both diseases respond to PGA. It is of interest, therefore, to contrast the initial serum carotene levels of a group of patients with sprue with a group of patients with pernicious anemia (fig. 3). These

findings are similar to those of Cayer and coworkers ('45, '46). Considered together with the normal-to-high glucose tolerance curves (Johnsson, '22; Hanes, '46) and the absence of the deficiency pattern on x-ray examination (Golden, '41) in pernicious anemia these findings indicate that the presence of the gastrointestinal defects is outstanding in sprue, but that this is not the case in pernicious anemia. The gastrointestinal abnormalities are not, then, secondary to macrocytic anemia.

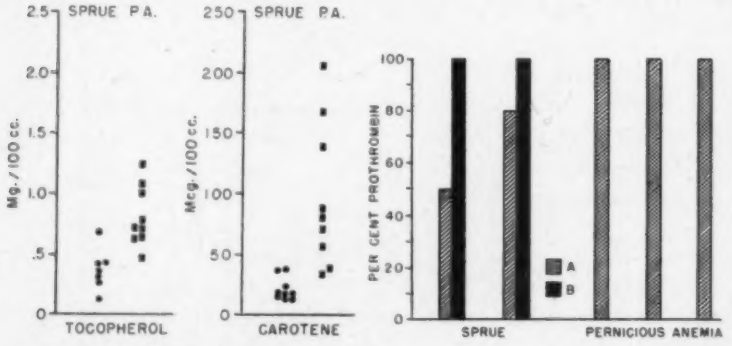

Figure 3

Fig. 3 Plasma tocopherol and serum carotene levels in sprue and pernicious anemia. The low initial carotene and tocopherol levels of patients with sprue are to be contrasted with the initial higher levels of patients with pernicious anemia in relapse. Carotene levels are included on 2 patients with sprue who were seen in other hospitals but are not reported in this paper.

Fig. 4 Plasma prothrombin levels in sprue and pernicious anemia. A, prothrombin concentration prior to therapy; B, prothrombin concentration after therapy with PGA. Note the contrast between the prothrombin concentrations in patients with sprue and pernicious anemia in relapse. The increase in prothrombin concentrations of the patients with sprue following therapy with PGA is not due to administered vitamin K.

The rise in serum carotene following therapy with PGA is illustrated in figure 2b and has been discussed in an earlier report (Darby et al., '47). This gradual rise may be attributed to the accumulation of carotene in the serum as the patient is better able to absorb the pigment. A sustained rise has been observed in 6 of the 7 remissions. The absence of a sustained carotene rise in 1 (J.H.) is partly explained

by the exceedingly low intake of carotene in his self-selected dietary (calculated to be 180 I.U. per day). In passing, the remarkable similarity of this increase in serum carotene content to the increased cholesterol levels in cases of sprue treated with liver extract (Fairley, '30) is to be noted.

Vitamin A absorption determinations following an oral dose of 200,000 I.U. of vitamin A have yielded data consistent with the interpretation that there was improved absorption in 2 of the 4 remissions in which repeated estimations were made. Two patients have continued to exhibit flat vitamin A tolerance curves despite clinical improvement. These individuals are persons with chronic sprue. One (P.B.) is an alcoholic; the other (J.H.) developed evidence of niacin deficiency following therapy with PGA.

Vitamin K

The hypoprothrombinemia which can be observed in sprue is attributed to a deficiency of vitamin K (Kark, Souter and Hayward, '40). Two of our patients have exhibited hypoprothrombinemia prior to therapy with PGA and this hypoprothrombinemia has returned to normal during remissions induced by PGA (fig. 4). By way of contrast, the prothrombin times determined on 3 patients with pernicious anemia in relapse are included in this figure. Again the absence of a secondary fat-soluble vitamin deficiency in pernicious anemia is impressive.

Vitamin E

Low plasma tocopherol levels have been reported in patients with sprue in relapse (Darby, Cherrington and Ruffin, '46). The decrease in serum tocopherol concentration during a relapse of sprue and the increase following therapy with pteroylglutamic acid are depicted in figures 2a and 2b. The contrast between the concentrations of fat-soluble vitamin E in the patients with sprue and in patients with pernicious anemia (fig. 3) is again noted.

Correlative data on many of the points discussed above are presented in figures 2a and 2b, which depict the course of patient J.D. during the 2 relapses and 2 remissions induced by PGA. These figures illustrate the rapidity with which alterations toward normal may occur in glucose tolerance following therapy and that this improved function may be maintained during a remission induced by PGA even after administration of the vitamin has been terminated. These figures also

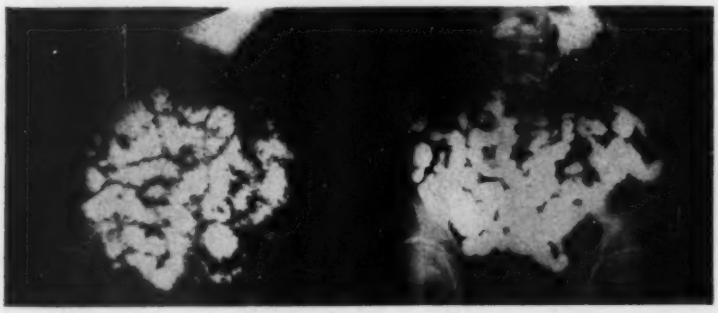

Fig. 5 Gastrointestinal x-ray pattern as revealed 1 hour after a barium meal, patient J.H. The film on the left was obtained prior to institution of therapy, the one on the right 2 months after the beginning of treatment with 5 mg PGA daily. Note the less abnormal appearance of the intestines upon the second examination.

indicate a reciprocal relationship between the carotene level in the serum and the amount of fat lost in the stool. The very close time association between the hematologic and gastrointestinal changes observed during remission and relapse is consistent with the hypothesis that these are 2 manifestations of a deficiency [4] disease.

X-ray changes in the gastrointestinal tract

X-ray changes of the so-called deficiency pattern type are present in sprue (Mackie et al., '35; Golden, '41). Beguerie and Spies ('46) have reported that these return toward nor-

[4] See footnote 2 page 645.

mal following the administration of PGA. We have obtained follow-up films on patients in 4 remissions induced by PGA. In 3 of these definite improvement in the pattern has occurred following therapy with PGA (fig. 5). Less pronounced improvement has been observed in the fourth. In none of our cases have the x-ray patterns returned completely to normal despite the otherwise excellent response to treatment. Other types of specific therapy likewise fail to restore the intestinal pattern completely to normal in cases of sprue (Mackie, Miller and Rhoads, '35; Golden, '41).

DISCUSSION

Our observations may be interpreted as indicating that the primary defect in sprue is a deficiency state [5] manifested by glossitis, macrocytic anemia and pancytopenia, and impaired gastrointestinal function. This primary deficiency is corrected by pteroylglutamic acid. The simultaneous correction of several secondary deficiencies is also indicated by the data presented in this report. The numerous interrelationships of pteroylglutamic acid with other vitamins and food factors are a most remarkable clinical example of the interrelationship of foodstuffs in man. The close similarities to the multiple deficiency states (Daft and Sebrell, '45) in the sulfa-treated rat are obvious.

Our data lead us to suggest that PGA will probably produce effects similar to those of liver extract on the serum cholesterol (Fairley, '30), on serum calcium (Fairley, '36), on the sodium deficiency in sprue (Black, '46), and on other secondary deficiencies which occur in the disease.

Many cases of sprue undoubtedly develop irreversible changes in the gastrointestinal tract (Golden, '41; Hanes, '42). In 1 instance, Golden ('41) has demonstrated degenerative changes in the intrinsic nerve plexuses which supply the intestinal wall. McCarrison ('21) observed similar changes in monkeys which apparently had vitamin M deficiency (Day, '44). The vitamin M deficient monkey may be regarded as the

[5] See footnote 2 on page 645.

experimental analogue of sprue (Darby and Jones, '45; Darby et al., '46b; Jones et al., '47). It is improbable that completely degenerated nerve plexuses can be restored. Therefore, the persistence of some degree of malfunction despite therapy may be expected, particularly in disease of long standing. The failure of liver extract therapy to correct *in toto* the various disturbances of gastrointestinal absorption may be thus explained. If the above hypothesis holds, complete restoration of normal function of the gastrointestinal tract may never occur. The relationship of the anatomical findings to the functional defects in absorption is not clear. Further studies are planned in an effort to define the relative roles of the degenerative changes in the plexuses and the possible alterations in mucosal permeability in vitamin M deficiency.

It should be emphasized again that any disease which results in malabsorption from the gastrointestinal tract may resemble the sprue syndrome (Hanes, '46). Although the clinical pictures are indistinguishable, it is to be expected that PGA will be ineffective in the treatment of any disease which does not result from a deficiency [6] of this vitamin. Therefore, the availability of this chemically well-defined factor may enable clinicians to separate the different disease entities which are now grouped together loosely as the sprue syndrome or idiopathic steatorrhea. The reported inefficacy of PGA in certain cases of celiac disease (Davidson et al., '47) may indicate that this disease in children is on a different etiologic basis than is sprue in the adult. In this connection it is well to recall the similarity of the gastrointestinal x-ray pattern in celiac disease in children and sprue in adults to that of the very young normal infant (Golden, '41).

In the discussion of these findings we have adopted the convention of interpreting all of these data in terms of changes in gastrointestinal absorption. A critical appraisal of the evidence in the literature upon which this practice is based makes it seem likely that it is at least partially correct.

[6] See footnote 2 on page 645.

However, other interpretations are possible; for example, the observed changes in glucose tolerance and vitamin A tolerance could be explained on the basis of altered hepatic function or a combination of altered intestinal absorption and hepatic storage or release as has been observed for vitamin A in rats (McCoord et al., '47) and for vitamin A and carotene in the human with infective hepatitis (Harris and Moore, '47). Furthermore, it is well established that rats treated with sulfonamides manifest several vitamin deficiencies. The usual interpretation of these findings is that the sulfonamide inhibits the synthesis of the vitamins by the flora of the gastrointestinal tract. In the sulfa-treated rat there occurs a decrease in the coliform organisms in the gastrointestinal tract. These organisms are able to synthesize a number of vitamins (Daft and Sebrell, '45). In unpublished observations made in collaboration with Dr. John Buddingh, we have observed that the fecal flora of our patients with sprue is characterized by a decreased number of coliform organisms and an increased number of slow and non-lactose fermenting organisms. During therapy with PGA a normal flora seems to have been reestablished. It will require many careful studies over long periods of time to define the relative roles of altered gastrointestinal absorption, metabolism and gastrointestinal synthesis in the pathogenesis of the manifold deficiencies observed in sprue.

SUMMARY

Observations on 6 patients with sprue during 7 remissions following therapy with PGA have been summarized. Each remission has been characterized by relief of the glossitis, regeneration of the lingual papillae (fig. 1), cessation of the diarrhea, and gain in weight, as well as hematologic improvement (figs. 2a and 2b). In 5 instances a return toward the normal glucose tolerance following therapy was demonstrated. A gradual increase in the serum carotene concentration was observed in 6 of the remissions and improved vitamin A absorption and increased prothrombin concentration (fig. 4) demonstrated in 2 instances each. Increased concentrations of

plasma tocopherol have been observed in 2 cases, and a decreased loss of fat in the stool has occurred in the 1 patient who was studied repeatedly (figs. 2a and 2b).

These findings are compatible with an interpretation that PGA exerts a favorable influence on the primary gastrointestinal disorder in sprue and thereby permits the correction of many secondary deficiencies which occur. Other possible interpretations are indicated.

Chronic sprue responds less completely to PGA than does the more acute disease.

As further evidence for the effectiveness of PGA in sprue, 2 patients have relapsed when therapy with PGA was withheld. All of the manifestations of the disease reappeared (figs. 2a and 2b).

Sprue and pernicious anemia differ in that gastrointestinal defects are pronounced in sprue.

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MINERAL METABOLISM STUDIES IN DAIRY CATTLE

I. THE EFFECT OF MANGANESE AND OTHER TRACE ELEMENTS ON THE METABOLISM OF CALCIUM AND PHOSPHORUS DURING EARLY LACTATION [1]

J. T. REID, K. O. PFAU, R. L. SALSBURY, C. B. BENDER AND G. M. WARD [2]

New Jersey Agricultural Experiment Station, Sussex, New Jersey

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The increasing capacity of the dairy cow for milk production has suggested to many investigators the possible need of supplying mineral elements to the cow in quantities greater than that provided by home-grown feeds. Numerous studies have resulted in the drawing of variable conclusions in regard to an adequate calcium and phosphorus supply for lactating cows. Generally the requirements are thought to be approximately 10 gm each daily, of calcium and phosphorus for a 1000-pound milking cow and an additional allowance of 0.9 gm calcium and 0.7 gm phosphorus per pound of milk produced as proposed by Huffman ('34).

Numerous balance trials have demonstrated the occurrence of negative calcium and, somewhat less frequently and for shorter periods of time, negative phosphorus balances during the early portion or peak of lactation. Ellenberger and associates ('31 and '32) and Huffman et al. ('30a) studying cal-

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cium and phosphorus metabolism during the gestation, lactation and dry periods found negative balances during peak production followed by a replenishing of the stores of these elements during the drying-off and dry periods. This has been considered to be a normal cycle during which the importance of the dry period was emphasized (Bohstedt, '42 and Hart et al., '31).

A series of extensive investigations made by Hart and associates to determine some of the dietary factors influencing the assimilation of calcium in cows and/or goats dealt with calcium ('23 and '27a), phosphorus ('23), cod liver oil ('27b and '29a) and irradiated yeast ('30) supplementation; the feeding of green grasses ('23a, '27a and '31), sun-cured ('29b, and Steenbock et al., '25) and weathered hays ('25); the feeding of glucose ('31) and hydrochloric acid ('31) and the exposure of animals to direct sunlight ('26 and '27a) and other ultraviolet light radiations ('24, '26 and '27c). These workers concluded from their experiments that there are factors other than vitamin D operating in the absorption of calcium from the intestinal tract of the cow.

There are no reports found in the literature which would indicate that manganese, iodine, copper, cobalt, iron or zinc influences the assimilation of calcium in lactating cows. Palmer et al. ('28), however, demonstrated that a high magnesium ration decreased the assimilation of calcium and phosphorus in cows. The effect was most marked when the phosphorus content of the ration was low. More recently, Huffman and coworkers ('30b) showed that the inclusion of 3 to 5% of magnesium salts in the rations of calves maintained on a low calcium ration either had no effect at all or slightly improved the retention of calcium and phosphorus. These investigators (Huffman and Duncan, '35) later demonstrated that magnesium improves calcium and phosphorus metabolism and bone calcification in dairy calves and described this function as a vitamin D-sparing effect.

The ingestion of beryllium salts was shown to lead to a type of rickets caused by the formation of insoluble beryllium phos-

phate in the intestinal tract (Kay and Byrom, '27). Apparently other metallic salts such as aluminum, thallium, barium, strontium and magnesium act similarly (Schmidt and Greenberg, '35).

Perosis, a disease indicated by slipped tendons in chickens, was proven by Wilgus and associates ('36) to be caused by a deficiency of manganese. Other investigations, dealing with the role of manganese in calcium and phosphorus metabolism, demonstrated a favorable influence of this element on the development of bone in rats (Amdur et al., '45, and Wachtel et al., '43), pigs (Miller et al., '40) and rabbits (Smith et al., '44).

In a study conducted by Chornock et al. ('42) using rats, an increased calcium and phosphorus excretion resulting in negative balances was effected by increasing the manganese intakes.

Reports in the literature on the effects of various mineral elements upon the metabolism of calcium and phosphorus in dairy cattle are limited except in the case of magnesium.

This experiment was initiated to study the effects of supplemental calcium and manganese and other mineral element supplementation upon the metabolism of calcium and phosphorus in dairy cows during the early portion of lactation.

EXPERIMENTAL

During this investigation the metabolism of calcium and phosphorus was studied by the balance trial method. Three weekly balance trials were conducted at regular intervals with each of 8 Holstein and 4 Guernsey cows during the first 5 months of lactation.

The 12 cows comprised 3 collection and 4 feed groups. The collection groups, composed of 4 animals from which excreta was collected while on trial simultaneously, were selected as nearly uniformly as possible relative to size, age, breed and stage of lactation. The feed groups received the following grain mixtures continuously during the 5-month feeding trial: group I, basal grain mixture (consisting of

ground corn, 30 parts; rolled oats, 30 parts; linseed meal, 28 parts; wheat bran, 11 parts and plain salt, 1 part); group II, basal grain mixture plus the addition of 3 pounds of $CaCO_3$ (c.p.) per 100 pounds of the basal grain ration, group III, basal grain mixture plus the addition of 3 pounds of a mixture of $CaCO_3$ (c.p.) and $MnSO_4$ (c.p.) (proportions of calcium and manganese same as in Mico [3]) per 100 pounds of basal grain mixture; group IV, basal grain mixture plus the addition of 3 pounds of Mico [3] per 100 pounds of basal grain mixture. Vitamin D supplements were not given to any of the groups.

The general feeding plan provided 1 pound of timothy-clover hay with 3.5 pounds of corn silage per 100 pounds of body-weight daily and grain in sufficient quantity to satisfy the Morrison feeding standards ('39) plus 10%.

The feces and urine were collected separately by attendants. Weekly composites of daily aliquots of silage, hay, water, milk, feces and urine were analyzed for calcium and phosphorus according to the official methods of the A.O.A.C. ('40).

The chemical methods used to determine the levels of certain blood and plasma constituents at intervals during the feeding trial are as follows: hemoglobin, Sanford, Sheard and Osterberg ('33); total plasma proteins, Looney and Walsh ('39); calcium, Clark and Collip ('25) and inorganic phosphorus, Fiske and Subbarow ('25). The red blood cell count and volume (hematocrit) were determined on the same blood samples according to the standard procedures.

The "percentage used" of calcium and of phosphorus was determined by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element taken in by the animal.

RESULTS AND DISCUSSION

Health and milk production

No differences were found in the general health of the animals of the 4 feed groups. It is not known, however, what

[3] Mico has the following percentage composition: calcium 33; magnesium 2; manganese 0.20; iron 0.20; iodine 0.045; copper 0.025; zinc 0.01 and cobalt 0.002.

would be the effects of the different supplements used or the lack of them over an entire lactation period or over the lifetime of an animal.

During the relatively short period of experimentation, no definite effects on the production of the cows were found as a result of calcium supplementation alone or of calcium plus trace elements supplementation; the control animals (group I) seemed to maintain their levels of production equally as well as the groups receiving the various supplements. The high milk production of group I as compared to the other groups is largely the reflection of the high production of cow 391 which possessed an inherently greater capacity for production than the other animals.

Blood picture

Although the hemoglobin levels, red blood cell counts and volumes of the blood of the animals of the 4 groups were not greatly different, these values were somewhat higher for groups II, III and IV than for group I (table 1).

Table 1 also shows no apparent group difference in the average levels of plasma calcium and phosphorus or in the levels of plasma proteins.

Calcium metabolism

Tables 2–5 show the average daily milk production, number of days in milk, and the intake, outgo, balance and percentages of calcium and phosphorus used for all animals of groups I, II, III and IV, respectively. It will be noted that each animal appeared on trial 3 times and that the metabolism period date indicates the sequence in which the trials were conducted. A general summary of group average calcium and phosphorus metabolism data and individual animal histories are given in tables 6 and 7, respectively.

Tables 2–5 show that during the 9 trials run on each group the following number of negative calcium balances were encountered in decreasing order of occurrence: group III, 9;

TABLE 1

Average blood picture of cows on different rations during feeding period (mean ± standard deviation).

GROUP	PLASMA			WHOLE BLOOD		
	Ca	Inorg. P	Total proteins	Hemoglobin	R.B.C.C.	R.B.C.V.
	mg %	*mg %*	*gm %*	*gm %*	*mill./mm³*	*%*
I	10.61 ± 1.09	5.41 ± 0.77	8.30 ± 0.75	9.97 ± 1.37	6.24 ± 0.85	27.85 ± 4.04
II	10.45 ± 0.86	5.12 ± 0.85	7.75 ± 0.36	10.70 ± 1.58	6.95 ± 0.63	31.14 ± 2.17
III	10.70 ± 0.83	5.63 ± 0.75	7.83 ± 0.39	10.70 ± 1.07	7.51 ± 1.11	29.84 ± 3.70
IV	10.47 ± 0.78	5.18 ± 0.89	7.90 ± 0.55	11.14 ± 1.63	7.65 ± 0.73	31.87 ± 2.81

TABLE 2

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain.

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	RATIO CA/P IN FEED	CALCIUM				PHOSPHORUS			
					Intake	Outgo	Balance	Used [1]	Intake	Outgo	Balance	Used [1]
			kg		*gm*	*gm*	*gm*	*%*	*gm*	*gm*	*gm*	*%*
391	11/24–11/30/45	56–63	25.36	0.93	61.75	72.93	—11.08	27.68	66.27	66.97	—0.70	27.81
566	12/4 –12/10/45	29–36	17.68	1.20	54.14	60.14	—6.00	23.72	45.00	47.44	—2.44	33.87
610	12/13–12/19/45	45–52	17.77	0.92	42.00	50.61	—8.61	42.65	45.76	47.02	—1.26	39.70
391	1/6 – 1/12/46	100–107	23.85	0.83	59.53	62.98	—3.45	35.95	72.06	73.12	—1.06	25.21
566	1/16– 1/22/46	73–80	18.37	0.96	53.11	49.37	3.74	42.41	55.30	57.47	—2.17	36.32
610	1/25– 1/31/46	88–95	14.42	0.61	39.11	40.96	—1.85	42.63	64.35	63.38	0.97	25.34
391	2/14– 2/20/46	139–146	20.91	0.65	51.90	54.31	—2.41	38.48	79.13	69.13	10.00	36.03
566	2/23– 3/1 /46	112–119	17.59	0.60	38.11	45.35	—7.24	27.75	63.28	61.63	1.65	33.50
610	3/6 – 3/12/46	121–128	16.39	0.54	32.12	36.40	—4.28	54.84	59.08	61.68	—2.60	28.07

[1] The "percentage used" of calcium and phosphorus was calculated by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element ingested by the animal. In this derivation milk is considered as a body constituent.

TABLE 3

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with calcium carbonate.

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	RATIO CA/P IN FEED	CALCIUM				PHOSPHORUS			
					Intake	Outgo	Balance	Used [1]	Intake	Outgo	Balance	Used [1]
			kg		*gm*	*gm*	*gm*	%	*gm*	*gm*	*gm*	%
529	11/24–11/30/45	56–63	13.80	2.34	92.48	95.27	—2.79	10.55	39.45	42.47	—3.01	16.97
552	12/4 –12/10/45	49–56	14.66	2.41	90.01	93.00	—2.99	13.09	37.39	33.39	3.52	39.75
765	12/13–12/19/45	40–47	18.07	2.27	117.56	122.66	—5.10	15.31	51.82	50.20	1.62	37.93
529	1/6 – 1/12/46	100–107	10.93	2.13	88.57	81.37	7.20	18.48	41.65	41.83	—0.18	20.87
552	1/16– 1/22/46	93–100	13.82	2.25	95.95	78.02	17.93	33.34	42.60	44.33	—1.73	25.37
765	1/25– 1/31/46	83–90	16.52	1.98	117.60	114.93	2.67	19.09	58.49	58.17	0.32	29.70
529	2/14– 2/20/46	139–146	7.30	1.92	85.60	86.02	—0.42	6.66	44.49	40.50	3.99	23.68
552	2/23– 3/ 1/46	132–139	13.84	1.81	87.78	88.29	—0.51	15.74	48.59	45.57	3.02	30.53
765	3/6 – 3/12/46	116–123	19.55	1.87	117.53	110.04	7.49	26.62	62.87	64.40	—1.53	33.48

TABLE 4

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with calcium carbonate and manganous sulfate.

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	MANGANESE ADDED TO RATION	RATIO CA/P IN FEED	CALCIUM				PHOSPHORUS			
						Intake	Outgo	Balance	Used [1]	Intake	Outgo	Balance	Used [1]
			kg	*gm*		*gm*	*gm*	*gm*	%	*gm*	*gm*	*gm*	%
530	11/24–11/30/45	50–57	18.23	0.41	2.22	119.95	123.99	—4.04	10.71	54.12	52.67	1.45	29.13
569	12/4 –12/10/45	34–41	12.22	0.18	2.34	75.27	85.99	—10.72	3.65	32.13	33.71	—1.57	32.37
733	12/13–12/19/45	57–64	16.03	0.40	2.18	105.52	117.99	—12.47	7.20	48.45	46.89	1.56	39.15
530	1/6 – 1/12/46	94–101	16.34	0.41	2.04	115.47	120.56	—5.09	8.55	56.53	54.43	2.10	27.89
569	1/16– 1/22/46	78–85	11.77	0.25	2.17	80.90	84.53	—3.63	10.99	37.22	38.88	—1.66	26.30
733	1/25– 1/31/46	100–107	14.35	0.44	1.87	103.62	115.62	—11.98	5.45	55.26	57.18	—1.92	24.85
530	2/14– 2/20/46	133–140	14.46	0.41	1.69	106.49	110.64	—4.15	9.29	62.72	54.11	8.61	33.90
569	2/23– 3/ 1/46	117–124	10.78	0.25	1.59	66.31	80.40	—14.09	—3.72	41.65	39.07	2.58	32.05
733	3/6 – 3/12/46	133–140	13.91	0.47	1.61	101.03	110.26	—9.23	8.23	62.84	60.49	2.35	28.77

[1] The "percentage used" of calcium and phosphorus was calculated by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element ingested by the animal. In this derivation milk is considered as a body constituent.

TABLE 5

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with Mico.

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	MANGANESE ADDED TO RATION	RATIO CA/P IN FEED	CALCIUM				PHOSPHORUS			
						Intake	Outgo	Balance	Used [1]	Intake	Outgo	Balance	Used [1]
			kg	*gm*		*gm*	*gm*	*gm*	%	*gm*	*gm*	*gm*	%
386	11/24–11/30/45	72–79	16.80	0.31	2.25	113.13	109.72	3.42	18.05	50.17	48.14	2.03	30.94
557	12/4 –12/10/45	50–57	16.23	0.30	2.23	103.91	106.30	—2.39	15.65	46.67	42.36	4.31	38.77
718	12/13–12/19/45	46–53	14.84	0.33	2.07	84.46	71.54	12.92	35.96	40.81	37.07	3.74	41.94
386	1/6 – 1/12/46	116–123	14.90	0.31	2.13	115.30	106.06	9.27	20.11	54.17	49.60	4.57	33.20
557	1/16– 1/22/46	94–101	13.86	0.30	2.21	103.87	87.83	16.04	29.67	46.90	49.13	—2.23	24.16
718	1/25– 1/31/46	89–96	13.46	0.45	1.88	103.33	90.08	13.25	28.46	54.82	54.64	0.18	23.52
386	2/14– 2/20/46	155–162	12.57	0.31	1.82	105.15	104.88	0.27	12.43	57.79	52.88	4.91	30.19
557	2/23– 3/ 1/46	133–140	12.21	0.27	1.63	77.94	81.58	—3.64	12.65	47.74	46.28	1.46	26.93
718	3/6 – 3/12/46	122–129	12.06	0.41	1.65	90 91	78.56	12.35	30.05	55.23	53.77	1.46	26.42

TABLE 6

Group average daily milk production, days in milk and calcium and phosphorus intakes, balances and percentages used (mean ± standard deviation).

GROUP	AVERAGE DAILY MILK	DAYS IN MILK	MANGANESE ADDED TO RATION	CALCIUM			PHOSPHORUS		
				Intake	Balance	Used [1]	Intake	Balance	Used [1]
I	19.14±3.55	88.3±37.0	.....	47.97±10.40	—4.58±4.34	37.35±9.76	61.14±10.98	0.27±3.93	31.76±5.28
II	14.28±3.67	93.3±36.0	.....	99.23±14.05	2.61±7.23	17.65±8.14	47.48± 8.74	0.67±2.51	28.70±7.66
III	14.23±2.39	91.9±36.2	0.36±0.10	97.17±18.58	—8.38±4.16	6.71±4.57	50.10±11.01	1.50±3.24	30.47±4.41
IV	14.10±1.72	100.9±37.3	0.33±0.06	99.78±12.66	6.83±6.61	22.56±8.63	50.48± 5.44	2.27±2.36	30.67±6.37

[1] The "percentage used" of calcium and phosphorus was calculated by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element ingested by the animal. In this derivation milk is considered as a body constituent.

group I, 8; group II, 5 and group IV, 2. Since the stage of lactation was similar for the animals of each group, table 6 embodying average figures (representing 9 balance trials) is presented by groups for comparison purposes. These data show that the percentages of calcium used by the 4 groups in decreasing order were: group I, 37.35 ± 9.76; group IV, 22.56 ± 8.63; group II, 17.65 ± 8.14 and group III, 6.71 ± 4.57. The control group cows receiving a low calcium ration utilized more of this element than the other groups, which substantiates the early work on this problem (Huffman et al., '30a).

It will be noted in table 6 that group III receiving the basal ration supplemented with $CaCO_3$ and $MnSO_4$ and milking an average of 91.9 ± 36.2 showed an average daily calcium balance of -8.38 ± 4.16 gm and $6.71 \pm 4.57\%$ used as compared to 2.61 ± 7.23 gm and $17.65 \pm 8.14\%$, respectively, for group II receiving the basal ration supplemented with $CaCO_3$ and milking an average of 93.3 ± 36.0 days. (When the data for animal 552 during metabolism period 1/16–22/45, which are not of comparable magnitude with the other data of group II, are eliminated, the balance and percentage of calcium used by this group are 0.69 ± 4.69 and $15.69 \pm 6.02\%$, respectively). The average daily calcium intake for the cows of groups III and II was 97.17 ± 18.58 and 99.23 ± 14.05 gm, respectively. Since the average daily milk production was 14.23 ± 2.39 kg and 14.28 ± 3.67 kg for groups III and II, respectively, the theoretical demand for calcium was similar for both groups, other than the requirements for maintenance (cow 569 was the only pregnant cow in groups II or III and was carrying a month-old fetus during her last trial period. The apparent depression of the metabolism of calcium in the animals of group III was attributed to the $MnSO_4$ ingested. It seems unlikely that the greatly reduced utilization of calcium can be explained by reference to the phosphatase enzyme system, as the primary effects of any acceleration of phosphatase activity by manganese as reported by Wiese et al. ('38) should be reflected in the metabolism of phosphorus. Although group III was slightly higher than group II

in the balance and percentage of phosphorus used, table 6 indicates that no appreciable group differences existed relative to the metabolism of phosphorus.

Upon a consideration of the calcium metabolism data for group IV, the problem is further complicated. A comparison of the data for groups III and IV in table 6 shows the average balance and percentage of calcium utilized by these groups

TABLE 7

Animal history.

ANIMAL NO.	GROUP NO.	BIRTH DATE	DATE OF LAST CALVING	LACTATION NO.	DATE BRED
		Holstein cow group			
391	I	2/ 5/37	9/29/45	7	not bred
529	II	2/18/42	9/29/45	2	not bred
530	III	2/20/42	10/ 4/45	2	not bred
386	IV	12/19/36	9/13/45	6	12/12/45
		Holstein first calf heifer group			
566	I	4/20/43	11/ 5/45	1	12/30/45
552	II	11/28/42	10/16/45	1	not bred
569	III	5/21/43	10/31/45	1	1/16/46
557	IV	2/14/43	10/15/45	1	1/ 5/46
		Guernsey cow group			
610	I	12/11/37	10/29/45	5	not bred
765	II	6/ 6/43	11/ 3/45	1	not bred
733	III	3/20/42	10/17/45	2	1/22/46
718	IV	9/27/41	10/28/45	2	1/16/46

to be -8.38 ± 4.16 gm and $6.71 \pm 4.57\%$ at an average of 91.9 ± 26.3 days in milk and 6.83 ± 6.61 gm and $22.56 \pm 8.63\%$ at an average of 100.9 ± 37.3 days in milk, respectively. The intake of calcium and manganese and the rate of milk production were similar in the 2 groups. During the last trial period cow 569 in group III was carrying a 4-week old fetus, while cows 386, 557, and 718 in group IV were carrying fetuses 8, 7 and 7 weeks old, respectively.

Although no explanation can be offered for the mechanism involved in the difference in calcium retention in groups III

and IV receiving similar amounts of calcium and manganese, these data together with a comparison of those for group II indicated that the increased calcium utilization was effected by the ingestion of the additional mineral elements of iodine, magnesium, copper, cobalt, zinc and iron by the animals of group IV.

Whether or not these results in some way are caused by the function of 1 or of several of the elements in the enzyme or endocrine systems is not known; however, these elements are known to be involved in several enzyme systems either as activators or as a portion of the enzyme.

Of interest is the reported observation that manganese is removed from solution by the insoluble portion of bone meal and Ca_3 $(PO_4)_2$ supplements and thus rendered unavailable to chickens (Schiable and Bandemer, '42). In the present study it is not known what was the fate of manganese added as $MnSO_4$ to the grain of the group III animals or that of the manganese contained in the Mico [4] fed to the animals of group IV. It is possible that these results could be better explained if the fates of the manganese were known in each case The manganese balances for this study will be determined later.

The results of this investigation offer an opportunity for conjecture as to their being effected by endocrines other than the parathyroid glands or by those influencing the parathyroids. Aub et al. ('29), Hunter ('30), Logan et al. ('42) and Pugsley and Anderson ('34) have demonstrated an increased calcium excretion following the administration of thyroid substance and/or during conditions of hyperthyroidism. Others (Kocher, '11 and Webster et al., '31) have shown in work with humans and rabbits that an excess of iodine may stimulate the thyroid to hyper-activity. Further work (Salter, '40) with small animals and humans indicated that the response of the normal thyroid to KI depends upon the route and duration of administration, state of the animal, the dosage used, the species and other factors.

[4] See footnote 3 on p. 664.

Salter ('40) stated that an increased thyrotropic activity of the pituitary was found during pregnancy and lactation. This may be associated with the negative calcium balances reported for cows during early lactation. Goormaghtigh and Handovsky ('35) and Nitschke ('33) attributed a thyrotropic action to vitamin D when administered in small doses and a depression of thyroid activity when large doses were given; this effect was thought to concern the metabolism of iodine. Campbell ('42) offered data supporting the belief that vitamin D tends to spare the parathyroid gland.

Although literature concerning the relationship between the sex glands, the anterior pituitary and mineral metabolism in the bovine is limited, Riddle and Dotti ('45) showed that the follicular hormone increases the serum calcium concentration of castrated animals of other species. These workers also showed that gonadotropins increase the plasma calcium level only in birds whose ovaries are sufficiently matured to produce estrogen, but are ineffective in males and ovariectomized fowl. This study did not determine whether the follicular or the luteinizing gonadotropin is chiefly concerned. Riddle and Dotti ('45) further demonstrated that estrogenic hormones (estrone, estradiol, estriol and diethylstilbestrol) have a specific ability to increase the bound calcium fraction in the plasma of doves, pigeons, and other fowl of both sexes. The latter investigation showed that these hormones are equally effective in normal, hypophysectomized, castrated, thyroidectomized and adrenalectomized pigeons. The androgens, however, did not affect the plasma calcium whatsoever. Progesterone showed only slight ability to increase the plasma calcium level.

The literature seems to indicate that various sex and other hormones may have a greater role in calcium metabolism, especially during early lactation, than was previously supposed, and that some of the mineral elements may indirectly affect the metabolism of calcium through some of the enzyme or endocrine systems.

Phosphorus metabolism

The data shown in tables 2–5 for the metabolism of phosphorus indicate that the balance and utilization of phosphorus were similar for all groups regardless of the supplement fed. However, groups III and IV which received supplemental manganese in addition to calcium showed slightly higher phosphorus balances than group I or II not receiving supplementary manganese, although only negligible differences existed in the percentages of phosphorus utilized. The number of negative phosphorus balances in order of increasing frequency were: group IV, 1; group III, 3; group II, 4 and group I, 6.

SUMMARY

A study was made of the effects of calcium and manganese and other mineral element supplementation upon the metabolism of calcium and phosphorus during the first 5 months of lactation of 8 Holstein and 4 Guernsey cows.

Calcium equilibrium was maintained most frequently in the group of cows receiving Mico [5] as the supplement to a basal ration of grain, corn silage and timothy-clover hay, and seemed to be attributable to the additional intakes of iodine, magnesium, copper, cobalt, zinc and iron supplied by this product.

Supplementation of the same basal ration with $MnSO_4$ in addition to $CaCO_3$ resulted in negative calcium balances in every case, whereas several positive balances occurred and higher percentages of calcium were used when the basal ration was supplemented with $CaCO_3$ alone. The marked depression of calcium metabolism appeared to be effected by $MnSO_4$ supplementation.

Phosphorus metabolism was not appreciably affected by the supplements used in this experiment.

[5] See footnote 3 on p. 664.

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THE EFFECTS OF CALORIC RESTRICTION ON SKELETAL GROWTH

PHILIP HANDLER, GEORGE J. BAYLIN AND R. H. FOLLIS, JR.
Departments of Biochemistry, Radiology and Pathology, Duke University School of Medicine, Durham, North Carolina

FOUR FIGURES

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It has long been recognized that the ingestion of inadequate quantities of a complete diet (chronic partial inanition) results in a cessation of bone growth. Recently, it was observed that the bones of young rats, on diets containing large quantities of lactose or galactose, failed to grow in length or width (Handler, '47). These animals ingested adequate quantities of a high calcium and phosphorus diet and, in fact, absorbed unusually large amounts of calcium from the intestinal contents because of the presence of the lactose (or galactose). However, these rats also failed to increase in weight because of a defective carbohydrate metabolism, suggesting that growth and calcification of the skeleton, like growth of soft tissues, are also dependent upon an adequate caloric supply and metabolism. The present paper describes the effects of simple caloric restriction with an otherwise adequate dietary regimen, on skeletal calcification and growth.

EXPERIMENTAL

The animals used were male rats of the Vanderbilt strain (Wolfe, Bryan and Wright, '38). The composition of the diets employed is given in table 1. It was arbitrarily decided to adopt, as the maximal limit of caloric restriction, approxi-

mately 50% of the *ad libitum* intake of normal controls of the same age and weight. By doubling the concentration of all dietary components save the sucrose of diet A, and then permitting the animals on diet B to eat only half as much as those on diet A, an intake of all essential nutrients (vitamins, minerals, proteins) equal to those of the *ad libitum* controls on diet A was assured. This procedure resulted in a caloric restriction of about 50% since diets A and B were virtually equicaloric. Diet C, when restricted to an intake equal to 50% of that of the controls on diet A, also resulted in a 50%

TABLE 1

Percentage composition of diets.

	A	B	C	D
Casein	25	50	25	40
Sucrose	67	34	59	48
Cod liver oil	4	8	8	6
Salt mixture[1]	4	8	8	6

In addition, each kilo of diet A contained thiamine chloride 4 mg, riboflavin 5 mg, pyridoxine 4 mg, calcium pantothenate 30 mg, nicotinic acid 10 mg, α-tocopherol 20 mg, naphthohydroquinone acetate 5 mg, *p*-aminobenzoic acid 50 mg, inositol 100 mg, choline chloride 250 mg. Each of these was doubled in diets B and C and increased 50% in diet D.

[1] Hubbell et al. ('37).

caloric restriction but the protein intake was also 50% of that of the controls. Diet D was so arranged that rats ingesting 75% as much food as *ad libitum* controls on diet A would receive identical supplies of all essential nutrients but only 75% of the caloric intake The adequacy of intestinal absorption of diets B, C and D under such circumstances remains an uncontrolled problem but there is no definite reason to suspect that it was faulty. The salt mixture used in these studies was one which provides rather more calcium and phosphorus than most salt mixtures used for such studies (Hubbell, Wakeman and Mendel, '37) and contains 21.5% calcium and 5% phosphorus. The dietary supply of these minerals, like that of the other essential nutrients was therefore adequately insured.

Such studies were made on a large number of rats but the data presented in table 2 are given only for 1 such experiment. The results, however, are quite typical of all the series. Rats of 2 ages were studied. Their size is indicated in the table. After 10 weeks on the various diets, the animals were anesthetized with nembutal given intraperitoneally, heparin was injected into the jugular vein, and blood collected by carotid section. With this procedure it is quite simple to obtain 5 ml of blood from a 150-gm rat. Plasma obtained in this manner was then used for determinations of calcium, phosphorus, alkaline phosphatase and total proteins. These data are presented in table 2.

TABLE 2

The effects of caloric restriction.

GROUP	NUMBER OF RATS	DIET	INITIAL WEIGHT	WEIGHT CHANGE	FOOD INTAKE	PLASMA CA	PLASMA P	PLASMA PHOSPHATASE	PLASMA PROTEINS
			gm	*gm*	*gm/day*	*mg %*	*mg %*	*B.U.*[1]	*gm %*
1	8	A	52	+168	9.6	9.1	7.3	15.2	7.2
2	8	D	53	+57	7.0	9.0	6.4	22.6	7.0
3	8	B	50	+3	5.0	8.7	6.1	28.4	6.6
4	8	C	53	—4	5.0	8.8	6.2	25.1	6.0
5	8	A	255	+82	16.3	9.2	6.8	12.4	7.4
6	8	B	251	—96	9.0	8.8	7.4	23.2	5.6
7[2]	6	A	237	+48	13.4	7.3	7.8	9.0	6.8
8[2]	6	B	241	—116	8.0	7.2	8.5	21.5	5.7

[1] Bodansky Units.

[2] Parathyroidectomized.

All animals were photographed by x-ray at the start of the experiment and again at its termination. Shown in figure 1 is a typical member of each group. From these plates it can be seen that the young rats, on a 50% caloric intake, not only failed in their generalized growth but also showed a complete cessation of skeletal growth as well. Considering also the fate of animals on the intermediate level of caloric intake, it appears that in young rats skeletal growth is roughly proportional to general body growth. The limited protein intake of group 4 did not affect this situation.

In contrast, considering the plates of the rats in groups 5 and 6, it appeared that in the larger rats, who were almost fully grown, a caloric restriction sufficient to produce a weight loss of 100 gm in 10 weeks (40% of their initial body weight) resulted in only slight interference with skeletal development.

Despite the impairment of lengthwise and transverse growth of the skeleton of caloric restricted young animals, no unusual changes were observed in the plasma calcium concentration in such animals. There did appear to be a definite tendency toward lowering of the serum inorganic phosphate concentration although this was not as marked as that seen in rickets and was not encountered in the larger animals. In contrast, in all series caloric restriction elicited a marked increase in the serum alkaline phosphatase activity.

Since identical increases in plasma phosphatase activity had been obtained in earlier series, it was then thought of interest to observe the effects of caloric restriction in parathyroidectomized rats. Because of the difficulties inherent in parathyroid ablation in rats, total thyroparathyroidectomies were performed under ether anesthesia. After a few days on a stock diet the rats were transferred to diets A and B to which 0.1% desiccated thyroid powder had been added. They are included in table 2 as groups 7 and 8. The animals on diet A did not grow quite as rapidly as did normal con-

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Figs. 1, 2 and 3 X-ray photographs of 1 typical member of each of the groups described in table 2. The photographs in the A series were taken on the first experimental day while those of the B series represent the same rats 10 weeks later. The photographs were taken under standardized conditions and developed and printed under identical conditions so that the figures reflect real differences in bone density.

1 Young rat, fed *ad libitum*.
2. Young rat, caloric intake ¾ of that of group 1.
3. Young rat, caloric intake ½ of that of group 1.
4. Young rat, caloric intake and protein intake ½ of that of group 1.
5. Large rat, fed *ad libitum*.
6. Large rat, caloric restricted.
7. Parathyroidectomized large rat, fed *ad libitum*.
8. Parathyroidectomized large rat, caloric restricted.

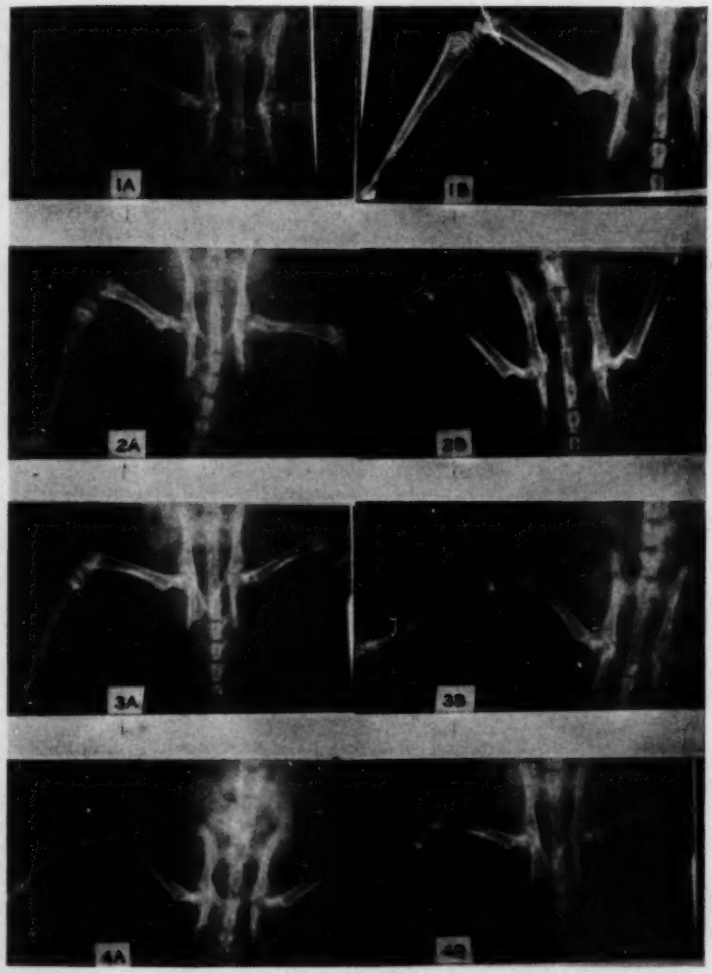

Figure 1

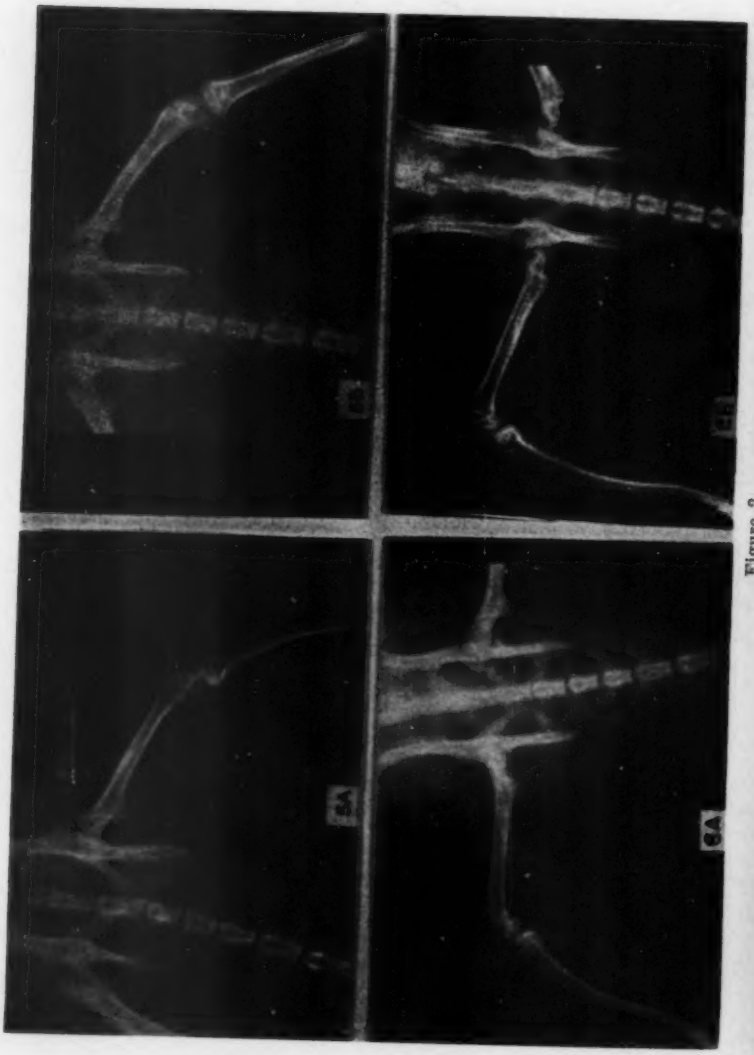

Figure 2

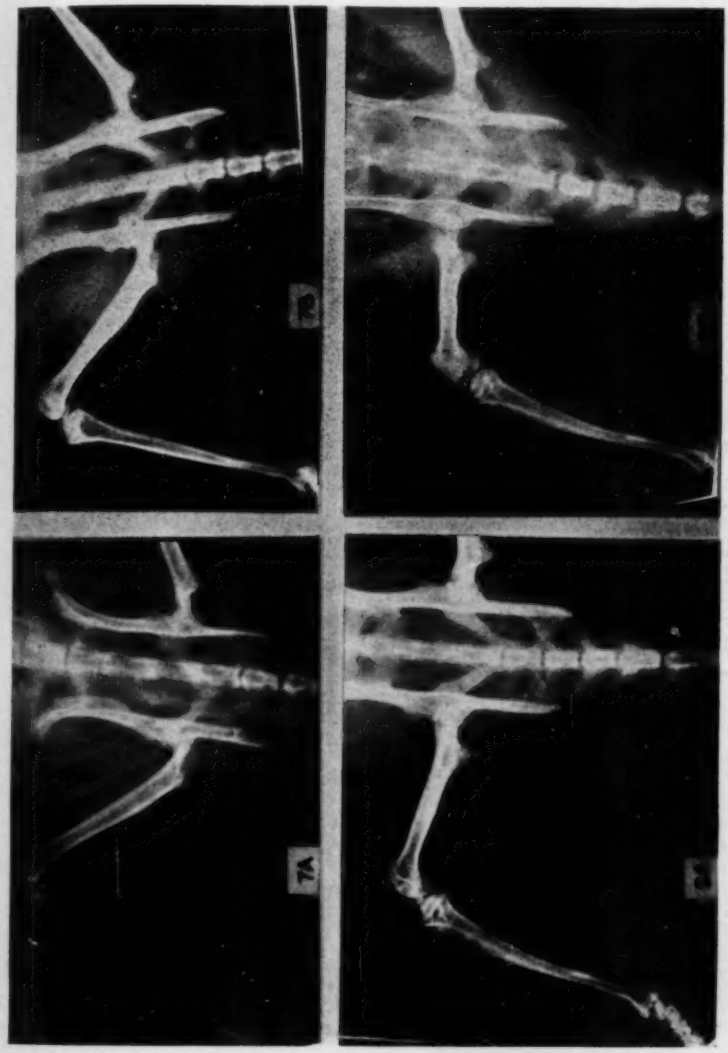

Figure 3

trols of this size while the animals on diet B lost somewhat more weight than did the unoperated animals in group 6. In all other respects, however, they resembled the unoperated animals. Like the rats in group 6 (caloric restricted large rats) the skeletons of the rats in group 8 continued to grow while the animals lost weight. As was to be expected they presented lower plasma calcium concentrations and higher phosphate concentrations than their controls. Of particular interest was the fact that in these parathyroidectomized, caloric restricted animals, also, there occurred a dramatic rise in serum alkaline phosphatase activity.

Representative femurs from rats in each group were fixed in formalin, and sections prepared for histological examination. In the younger animals there was found definite alteration in bone formation. There were fewer trabeculae; those that were present were stumpier than normal and the cartilage plate at the epiphyses was not growing actively. In addition, the cortices appeared thinner than in the control animals. There was no excess osteoid. In the older animals similar differences were observed but in lesser degree. All these changes are non-specific and resemble what is encountered in inanition which has been produced by various means. In summary, these changes are those which are usually observed in poorly growing or athreptic animals.

Fig. 4 X-ray photographs of the right, hind legs of typical members of each of the groups in table 2. The legs were dissected free and then photographed simultaneously on 1 plate. The numbers shown correspond to the group numbers of table 2, but the animals chosen are, in each case, different from those shown in figures 1–3.

1. Young rat, fed *ad libitum*.
2. Young rat, caloric intake $\frac{3}{4}$ of that of group 1.
3. Young rat, caloric intake $\frac{1}{2}$ of that of group 1.
4. Young rat, caloric intake and protein intake $\frac{1}{2}$ of that of group 1.
5. Large rat, fed *ad libitum*.
6. Large rat, caloric restricted.
7. Parathyroidectomized large rat, fed *ad libitum*.
8. Parathyroidectomized large rat, caloric restricted.

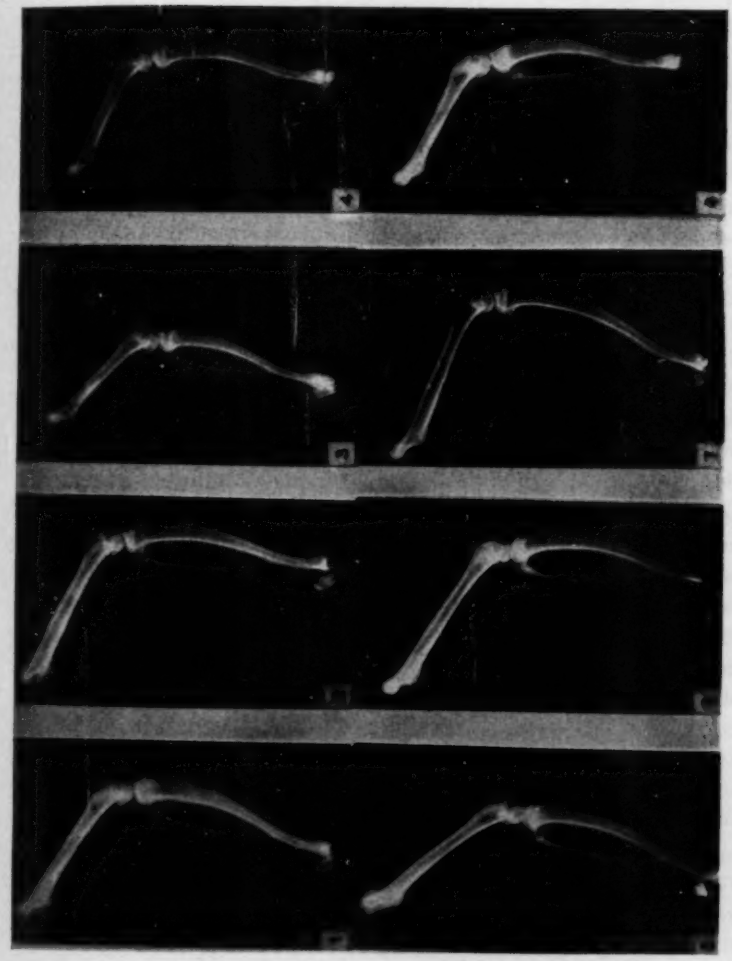

Figure 4

DISCUSSION

From the data presented herein it seems quite clear that caloric restriction results in impairment of skeletal growth and that this phenomenon is more marked in young rats than in those whose growth is almost complete. Since growth and remodeling of bone are functions of age, this observation is not unexpected. Moreover, since skeletal growth, like that of any other tissue, is dependent upon a cellular mechanism which requires an active carbohydrate metabolism (Gutman, Warrick and Gutman, '42) a disturbance in osteogenesis is not surprising. In a sense, these experiments serve as a control for comparison with the numerous previous studies of the effects of partial or complete inanition and indicate that the possibility of inadequate caloric supply must be considered whenever bone growth is being studied, independent of the supply of those nutrients which have a specific effect on bone formation such as ascorbic acid, vitamins A and D, calcium and phosphorus. These experiments certainly indicate a need for pair-fed controls whenever studies are made of osteogenesis in nutritionally deficient animals. It may be presumed that the classical picture of bone in inanition is more likely to be the result of an inadequate caloric supply than of any other single dietary factor. It would be of interest to determine whether this picture might be modified by the action of estrogens, which decelerate the normal destruction of bony trabeculae just beneath the cartilage shaft junction (Day and Follis, '41), or androgens, since both of these can induce positive calcium balance, particularly in osteoporotics (Reifenstein and Albright, '47).

At present it is not possible to interpret the results of the blood chemical findings in these animals in a definitive fashion. The plasma of all young, caloric restricted animals was found to have an essentially normal calcium concentration but diminished inorganic phosphate concentration and markedly elevated alkaline phosphatase activity. The plasma inorganic phosphate concentration of caloric restricted animals of the older age group was not essentially different from that of

their controls. This may be correlated with the fact that skeletal growth proceeded at the normal slow rate in these animals. However, they too presented elevated plasma alkaline phosphatase activity. It would seem then that this rise in phosphatase activity may not have been a reflection of metabolic events in the skeleton, but, rather, was the result of an altered hepatic metabolism under these circumstances.

Investigators of the effect of total starvation on serum alkaline phosphatase activity (Bodansky and Jaffe, '31; Weil and Russell, '40) have consistently encountered a fall of about 50% from normal values. However, these studies were, perforce, of brief duration and Wachstein ('45) has found that the alkaline phosphatase activity of the livers of rats and mice dying of starvation, determined histologically, is decidedly elevated. Since Oppenheimer and Flock ('47) have found that the increased hepatic alkaline phosphatase activity during the regenerative period following partial hepatectomy in the rat is accompanied by an increased plasma phosphatase activity it would seem that such phosphatase can escape from the liver to accumulate in the circulation. This possibility seems all the more likely when one considers that there is no other evidence of failure of biliary concentration in partially hepatectomized rats and the alkaline phosphatase activity of both normal and regenerating liver is largely bound to the chromatin structure in the nucleus or mitotic figures. Moreover, although no evidence is available which demonstrates a direct control by the parathyroid of bone or serum alkaline phosphatase, it does seem strange that parathyroidectomy should not have affected the level of plasma phosphatase activity in caloric restricted rats if the phosphatase were arising in the metabolically deranged bone. Considering these facts, it is felt by the authors that while the low plasma phosphate and high alkaline phosphatase concentrations in young caloric restricted rats are accompanied by osteogenetic disturbances, they are probably the consequence of an altered hepatic carbohydrate metabolism rather than of metabolic events in the skeleton.

SUMMARY

In young rats the rate of skeletal growth was roughly proportional to the rate of general body growth when caloric restriction was the limiting growth factor. Restriction of caloric intake to about one-half of that of *ad libitum* fed control animals, while ensuring an adequate supply of all other nutrients, resulted in a complete cessation of both skeletal and generalized body growth. Histologically the bones of such animals were identical with those usually found in "partial inanition." In larger, not quite completely grown rats, caloric restriction, sufficient to cause a loss of 40% of their initial body weight, only slightly decreased the rate of skeletal growth.

The plasma concentration of calcium was normal in all animals, while that of inorganic phosphate was below normal in the young caloric-restricted animals but not in the older rats. Caloric restriction in young rats, older rats and parathyroidectomized older rats resulted in a markedly elevated plasma alkaline phosphatase activity. However, it is thought that these latter changes in the caloric restricted rats are due to an altered hepatic carbohydrate metabolism rather than to the defective bone metabolism.

ACKNOWLEDGMENTS

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THE EFFECT OF ADDED THIAMINE ON GROWTH, VISION, AND LEARNING, USING IDENTICAL TWINS

E. CHANT ROBERTSON, C. M. TATHAM,[1] NORMA FORD WALKER AND M. REID WEAVER

The Hospital for Sick Children and the Department of Paediatrics, University of Toronto, under the direction of Alan Brown, M.D., F.R.C.P. (Lond.) and the Departments of Psychology and Zoology, University of Toronto

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Two reports have been published (Harrell, '43 and '46) which indicated that when half of the children living in an orphanage were given 2 mg of thiamine daily, in addition to their meals, their vision and their ability in a considerable number of psychological tests improved as compared with the other half of the children who received placebos and served as controls. Each child in the control group was matched as closely as possible with one receiving thiamine. For 2 periods, several days in length, all the foods eaten in the orphanage were weighed and the thiamine intake per child was calculated from tables. The estimated intake varied from 0.9 to 1.0 mg per child per day, but it was noted that "no deduction was made for possible excessive cooking loss."

PRESENT STUDY

In the present study, 44 pairs of identical or uniovular twins, living in their homes, were used as the test subjects. The twins were shown to be identical, that is, 1-egg pairs, by

[1] Swift Foundation research fellow.

the comparison of the color of their eyes and hair and by the study of their finger, palm and foot prints (this part of the study was carried out by Dr. Norma Ford Walker and Mrs. Reid Weaver). This method is accepted as reliable by geneticists. Identical twins provide subjects as closely matched as possible. After the initial examination was completed (see later), 1 member of each pair received 2 mg of thiamine and the other a placebo each day.[2] At the beginning of the study the children ranged from 7⅓ to 15½ years in age and were in grades 1 to 9 in school.

CALCULATION OF THIAMINE IN DIET

The eating habits of the 2 members of each pair were nearly always found to be closely similar when the mother and the individual children were questioned on this subject, on 3 or more occasions. In April and May, when the children had been receiving the tablets about 4 months, each mother was asked to keep a week's record of all food eaten by each of the twins. As the food was not weighed, the amounts were recorded in common household measures. A calculation of the thiamine content of the food eaten was then made, using the Table of Food Values recommended for use in Canada (Nutrition Division, Department of National Health and Welfare, '46). The results obtained are shown in table 1. Nine of the 24 pairs of twins were eating approximately the recommended daily allowances of thiamine (Food and Nutrition Board, National Research Council, '45), as is shown in column 3 of this table, headed "middle range." Twelve of the 24 pairs were eating 20 to 40 % less than the recommended allowances. In this case, the calculated intakes are shown in column 2, under the heading of "low." Three of the pairs were eating more than the recommended amounts (column 4). It was impossible, under the circumstances, to carry out the numerous determinations necessary to assay chemically the amount of thiamine in the food.

[2] Tablets donated by Mead Johnson and Co.

No correlation could be found between the estimate of the thiamine content of the diets and the effectiveness of the thiamine supplement, as measured by the tests listed below. The economic positions of the families varied greatly. Eight pairs were living in very poor homes and unfortunately 5 of these had to be discarded because the parents did not co-operate in giving the tablets regularly. Three other pairs, living in good homes, also had to be dropped for the same reason. Of the 36 pairs who took their tablets with sufficient regularity, 5 pairs were quite well-to-do, 28 pairs were apparently in fair to moderately good financial circumstances and 3 pairs were in poor homes.

TABLE 1

AGE IN YEARS	CALCULATED DAILY THIAMINE IN FOOD EATEN BY 24 PAIRS OF TWINS			AMOUNTS RECOMMENDED BY FOOD AND NUTRITION BOARD, N. R. C.
	Low	Middle range	High	
	mg	*mg*	*mg*	*mg*
8	0.7	0.9	2.0	1.0
9	0.7	0.8, 0.9		1.0
10	0.7	1.1, 1.1		1.2
11	0.8	1.0	1.3	1.2
12	0.9	1.1		1.2
13 (boys)	0.9, 0.9, 1.2		1.8	1.5
13 (girls)	1.0, 1.0	1.3		1.3
14 (boys)	0.9, 1.0			1.5
14 (girls)		1.3		1.3

PROCEDURE — INITIAL TESTS PRIOR TO EXPERIMENTAL PERIOD

Thanks to the kind co-operation of the school staffs, facilities were provided which made it possible to carry out all the tests in the schools. Initial tests, prior to the experimental period, were made in order to compare the physical and psychological status of the members of each pair and also to exclude any pair in which 1 twin was severely handicapped. These tests included a physical and neurological examination, the determination of weight (indoor clothes, no shoes), standing height (no shoes), a rough test of hearing and a careful test of the vision of each eye alone and of both eyes together.

For the latter a Snellen chart,[3] on which the illumination could be kept constant, was used. This apparatus included a light meter, set in a fixed position, which measured the light reflected from the chart and a rheostat for regulating the intensity of the light emitted by the 2 rows of frosted bulbs that were mounted on the right and left sides of the chart. Care was also taken to ensure good illumination in the room where the test was done. The child's vision was scored by allowing a specified mark for each letter read correctly at a distance of 20 feet — the marks becoming progressively larger as the letters became smaller.

The following 8 tests were given by the psychologist taking part in the study. They took 1 whole school day to administer, and in order to reduce the effect of fatigue, the time was spread over 2 days. For example, a pair of twins examined one morning would receive the remainder of the tests the following afternoon.

PSYCHOLOGICAL TESTS

NAME OF TEST	ABILITY TESTED
1. *Individual test of intelligence* Binet, form L	Intelligence
2. *Test of reasoning*,[4] *Grades 4–8* (Simple problems)	Reasoning
3. (a) *Dominion Test of Reading* Vocabulary (grades 2 and 3)	Reading
(b) *Gates Reading Survey* Vocabulary (grades 3–10)	Reading
4. (a) *Arithmetic achievement tests*[5] (For grade 2 and for grade 3)	Arithmetic
(b) *Dominion Arithmetic Test* Fundamental operations (grades 4–8)	Arithmetic
5. *Test of Rote Memory*[6] Memorizing meanings of 18 entirely strange words; testing immediately thereafter.	Immediate memory

[3] We are indebted to the Department of Physiology, University of Toronto, for the loan of this apparatus, which had previously been used in the Royal Canadian Navy.

[4] Prepared by Dr. John Long, Ontario College of Education.

[5] Prepared by Dr. A. J. Phillips, Ontario College of Education.

[6] Courtesy of Dr. Ruth F. Harrell, Norfolk, Virginia.

NAME OF TEST	ABILITY TESTED
6. *Testing of Faces and Names*[8] Given 5 short measured times to memorize names of 20 faces during initial testing. Given no chance to refresh memory subsequently, but shown faces and asked to name them.	Retention or forgetting over prolonged period
7. *Test of Code Substitution*[7] Code at the top of sheet consisting of 5 geometric symbols with a different digit under each. Child to put correct digits under symbols printed on large sheets in specified time.	Speed, accuracy
8. *Test of Nail Sorting*[8] Filling the greatest number of standard holes with 3 nails, head up, in a given time.	Manual dexterity

RESULTS OF INITIAL TESTS

As soon as the initial tests were completed, the senior author assigned one twin to the experimental group, to receive thiamine, and the other twin was placed in the control group. The psychologist, the twins, the parents and the teachers did not know which child was receiving the thiamine.

The average scores of the 2 groups at the beginning of the experimental period were very similar in all the tests. The results of a number of the tests are shown in table 2.

TABLE 2

Averages of initial tests in 36 pairs of twins.

	CONTROL	EXPERIMENTAL
Binet (I.Q. points)	104.6	103.6
Height (inches)	55.9	55.7
Weight (pounds)	78.5	77.4
Code substitution	201.4	201.3
Nail sorting	84.1	82.3

The intelligence quotients ranged from 76 to 158 points. The 2 individuals in 7 pairs had the same I. Q. (although not due to passing identical items), those in 16 pairs showed less than 5 points difference, and those in 13 pairs more than this.

As far as the individuals making up the pairs were concerned, their abilities in the special tests used varied much

[7] Obtained from Teachers College, Columbia University, New York.

[8] Obtained from the Department of Psychology, University of Toronto.

more than was anticipated. The psychological differences found in the pairs will be reported elsewhere.

EXPERIMENTAL PERIOD

(A) Short term experiment — first 4½ months

As soon as the initial tests were completed the twins were started on therapy. The placebos were made of lactose of identical shape and size as the thiamine-containing tablets, but were colored a pale pink. Each child had his own box of counted tablets, with his name clearly marked on it and a red seal as well in the case of the placebos. The importance of giving each twin his own tablet once a day, preferably after breakfast, was stressed by the senior author when the mother was visited, and she was given a calendar on which to mark each day on which tablets were given. The mothers were visited once a month by the senior author, when the number of tablets remaining was counted, the calendar checked, and more tablets left with her. The mothers, who were all very interested in their twins, were extremely cooperative. They had been told that 1 twin would receive more thiamine per day than the other. This, of course, included the thiamine eaten in their food. The short-term test covered an all-over testing period of 7 months for the examiners (November to June), and of 4½ months (the average figure) for each pair of twins. During this time, the average number of tablets forgotten was 7. Seven pairs of twins remembered to take the tablets every day and the maximum forgotten by any pair was 29. The psychological tests numbered 5, 6, 7 and 8 were re-administered every 3 weeks during this 4½ months of therapy. At the end of the short-term test, the whole battery of physical and psychological tests were repeated. At this time, form M of the Binet test was used, and the alternative forms of tests 2, 3 and 4.

Results of short-term test (first 4½ months)

These results are shown in table 3. The scores obtained were subjected to statistical analysis. When 2 trials only had

been given, the level of significance of the superior or inferior gains made by the thiamine-fed children was measured by the t-statistic. Where more than 2 trials were given, the method of analysis of variance was used. The latter provides an F-statistic for the significance of the differences between the trends of the scores of the experimental and the control

TABLE 3

Superior or inferior gains of thiamine-supplemented children in short-term test.

Subjects — 36 pairs Duration — 136 days (average)
F — Statistics and t — Statistics with level of significance.

NAME OF TEST	T_0	LEVEL OF SIGNIFICANCE OF T_0	F_1	LEVEL OF SIGNIFICANCE OF F_1 — 5%
Intelligence — Binet	— 0.772	40%		
Reasoning — (Gr. 4–8)	0.199	80%		
Reading — vocabulary (Gr. 2)	— 0.612	60%		
Reading — vocabulary (Gr. 3–10)	— 0.073	90%		
Arithmetic (Gr. 2 and 3)	— 1.498	20%		
Arithmetic (Gr. 4–8)	— 1.596	10%		
Rote memory			0.773	2.43
Code substitution			0.462	2.43
Manual dexterity			2.336	2.43
Faces and names (retention)			2.159	3.09
Height	2.187	5%		
Weight	2.260	Between 2 and 5%		
Eyesight (left eye)	0.925	40%		
Eyesight (right eye)	1.108	30%		
Eyesight (both eyes)	0.902	40%		

— Indicates tests in which control group made superior gains, as compared with thiamine-supplemented group.

groups. (F_1 indicates the significance of differences between trials.)

From table 3 it is seen that the experimental group (receiving thiamine) made superior gains in height (5% level) and weight (between 2% and 5% level). This means that in only 5, or 2 times, as the case may be, out of 100 could these

superior gains be attributed to chance. The 5% level of significance is not considered conclusive but only doubtful. In the tests of Nail Sorting (manual dexterity) and Faces and Names (retention or forgetting over a prolonged period), the experimental group made superior gains which almost reached the 5% level of significance. In reasoning, rote memory, code substitution, and eye-sight, the superiority of the experimental group was very slight and not significant statistically. In intelligence, reading, and arithmetic, the control group was slightly superior but this again was not significant statistically.

(B) Long-term test (9 months' duration)

The long-term test included the short-term one described above plus an extension, over the summer holidays, of roughly 4½ months. During the summer it was not possible to maintain close contact with the families and as a result 11 pairs of twins stopped taking the tablets or took them very irregularly. The long-term study, therefore, comprised 25 pairs of twins. During the 9 months of this test, the average number of tablets missed was 10, the maximum number forgotten was 29, and 2 pairs took them every day. At the end of the 9 months, the complete battery of physical and psychological tests was readministered, using the original forms. The results are shown in table 4.

It is seen that in reading and arithmetic the older children (grades 3 to 10) in the control group made slightly greater gains than those given the thiamine supplement. These gains were not significant statistically. In the other tests, the experimental group (thiamine-supplemented) showed slightly greater gains, which again were not significant statistically. Why the control group largely "caught up" on the thiamine-supplemented group, during the summer, is not clear. It has been reported that American children grow more in weight during the summer and autumn than in the rest of the year. Also their diets, during the summer and autumn, contain more fruits and vegetables, which would increase the thiamine in-

take to some extent. Previous investigators (Ross and Summerfeldt, '35), have shown that the addition of vitamin B complex improves the growth of children from 5 to 14 years of age.

TABLE 4

Superior or inferior gains of thiamine-supplemented children in long term test.

Subjects — 25 pairs Duration — 273 days (average)
F — Statistics with levels of significance.

NAME OF TEST	F_1	LEVEL OF SIGNIFICANCE OF F_1 — 5%
Intelligence — Binet	1.613	3.19
Reasoning (Gr. 4–8)	1.073	3.26
Reading — vocabulary (Gr. 3–10)	— 2.62	3.25
Reading — vocabulary (Gr. 2)	0.51	4.46
Arithmetic — (Gr. 4–8)	— 1.75	3.26
Arithmetic — (Gr. 2 and 3)	0.48	4.10
Rote memory	0.529	2.29
Code substitute	0.624	2.29
Manual dexterity	0.345	2.29
Faces and names (retention)	1.203	2.46
Height	0.483	3.19
Weight	1.148	3.19
Eyesight (left eye)	0.83	3.20
Eyesight (right eye)	1.39	3.21
Eyesight (both eyes)	0.61	3.20

— Indicates tests in which control group made superior gains, as compared with thiamine-supplemented group.

CONCLUSIONS

1. The administration of 2 mg daily of thiamine for 4½ months to half of 36 pairs of identical twins resulted in improvement in weight and height gains, and in manual dexterity and prolonged memory test scores, that were of questionable statistical significance. The tests used for measuring vision, intelligence, reasoning, arithmetic, rote memory and code substitution gave results of no statistical significance.

2. Twenty-five pairs of the twins were kept in the study for 4½ months longer. When they were again tested at the end

of this period, that is, after 9 months of therapy, no gains of statistical significance were evident in any of the tests.

ACKNOWLEDGMENTS

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STUDIES ON THE REQUIREMENT OF THE CHICK FOR TRYPTOPHANE

M. C. WILKENING,[1] B. S. SCHWEIGERT, P. B. PEARSON AND R. M. SHERWOOD

Department of Biochemistry and Nutrition and Department of Poultry Husbandry, Agricultural and Mechanical College of Texas, College Station, Texas

ONE FIGURE

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Since the avian species' diet consists largely of cereal grains and their by-products, and since the limited amount of tryptophane present in most cereal grains is recognized, the need for considerable information on the tryptophane requirement by various members of the avian species is emphasized.

Work on the tryptophane requirement of the chick has been reported by Almquist and Mecchi ('41) and Grau and Almquist ('44). Recent studies by Briggs ('45) and Briggs, Groschke and Lillie ('46) demonstrated that the tryptophane requirement was influenced by the level of nicotinic acid present in the ration.

In the present work, experiments were conducted to obtain additional information on the tryptophane requirement of the chick when optimum levels of nicotinic acid were supplied in the ration. An improved basal ration was developed in which the protein was supplied by oxidized casein (Toennies, '42) and gelatin supplemented with the sulfur amino acids. This ration when properly supplemented with tryptophane supported growth which was comparable to that obtained with a practical chick starter or with diets containing untreated

[1] Present address: Research Laboratory, Security Mills, Knoxville, Tennessee.

casein. An independent, second method for determining the tryptophane requirement of the chick involved the substitution of casein for oxidized casein in the basal ration. With this method graded levels of casein were fed as the source of tryptophane. The tryptophane requirement of the chick was then established from the minimum level of casein fed that supported optimum growth and efficiency of gain (gm gain/gm feed consumed). The amount of tryptophane contributed by this level of casein was determined by microbiological analysis.

EXPERIMENTAL AND RESULTS

Care of chicks. The room in which the chicks were housed was equipped with steam heat and an air cooling unit. The temperature of the room was maintained between 27 and 30°C. The chicks were confined in electrically heated, wire floored brooders. A thermostat controlled the temperature of the brooders to approximately 35°C.

Unsexed, day-old New Hampshire × White Leghorn crossbred chicks and White Leghorn chicks were used in these experiments. They were wing banded, weighed, and fed a practical chick starter for 10 days. Food and water were given *ad libitum*. The chicks were weighed at 5-day intervals. After 10 days the chicks whose weight deviated the greatest from the mean were discarded. Ten chicks were then selected for each experimental group on the basis of their weight, gain, and vigor so that the average weight and average gain were the same for all groups. The experiments were conducted for a period of 2 weeks, and the chicks were weighed on the fifth, tenth and fourteenth days. Growth obtained with diets to which various levels of tryptophane had been added was the major criterion used for establishing the tryptophane requirement. The efficiency of gain was also used as an index of the tryptophane requirement.

Composition of ration. The basal ration had the following percentage composition: oxidized casein 12, gelatin 10, salts IV (Hegsted et al., '41) 5, corn oil 5, fish solubles (dry basis)

2, *l*(-)cystine 0.5, *dl*-methionine 1.0, fortified cod liver oil (400D-3000A per gm) 0.75, and corn starch to 100. Each 100 gm also contained the following amounts of vitamins (in mg): thiamine 0.60, riboflavin 0.66, pyridoxine 0.66, calcium pantothenate 2.2, nicotinic acid 5.0, inositol 100, choline 200, *p*-aminobenzoic acid 0.2, biotin 0.02, pteroylglutamic acid 0.2, 2-methyl-1,4-naphthoquinone 2.0, and mixed tocopherols 10.0. The vitamins and graded levels of tryptophane were added at the expense of the starch.

Adequacy of the ration. Previous workers, who employed purified diets in which casein and gelatin were the sole sources of protein, fed at least 18% casein. The possibility of using a diet containing 12% oxidized casein was investigated since a study of the amino acid composition of the proposed diet revealed that it apparently supplied all the essential amino acids in adequate quantities with the exception of the sulfur amino acids, tryptophane, and possibly threonine (table 1). Preliminary experiments were then conducted to compare the growth-promoting properties of the 12% oxidized casein diets, supplemented with an adequate amount of tryptophane, with a practical chick starter diet and diets containing 12 and 18% casein. From the results obtained in these feeding trials (table 2) it can readily be seen that the growth response of the chicks was the same for all rations. As a result of these experiments it was concluded that the 12% oxidized casein diet, when properly supplemented with tryptophane, was a reliable ration supplying adequate quantities of all necessary nutrients essential for the normal growth of the chick. Consequently, the diet which contained 12% oxidized casein [2] was used in all subsequent experiments.

These experiments demonstrated that the 12% casein diet supported maximal growth when supplemented with cystine, methionine, and tryptophane. This observation formed the

[2] The procedure of Toennies ('42) was modified in order to produce larger quantities of this material. Details of this procedure may be obtained from a thesis submitted by M. C. Wilkening as a partial fulfillment of the requirements of a Master of Science degree in Biochemistry and Nutrition, July, 1947.

TABLE 1

The amino acid composition of the basal ration and the reported requirement of each amino acid for the chick.

AMINO ACID [1]	AMINO ACID SOURCES			TOTAL AMINO ACID CONTENT	REPORTED REQUIREMENT AND REFERENCE
	12% oxidized casein	10% gelatin	2% fish solubles (dry basis)		
	gm	*gm*	*gm*	%	%
Histidine	0.34	0.06	0.08	0.48	0.15 [2]
Arginine	0.47	0.91	0.06	1.44	1.0 [3]; 1.2 [3]
Lysine	0.92	0.58	0.06	1.56	0.9 [3]; 1.1 [3]
Leucine	1.19	0.35	0.06	1.60	1.5 [4]
Isoleucine	0.67	0.17	0.04	0.88	0.5 [4]
Valine	0.80	0.27	0.04	1.11	0.7 [4]
Methionine	0.00	0.06	0.02	0.08	0.6 [3]; 0.5 [3]
Threonine	0.44	0.20	0.03	0.67	1.0 [3]
Tryptophane	0.003	0.001	0.004	0.008	0.5 [3]; 0.25 [5]
Phenylalanine	0.71	0.23	0.03	0.97	0.5 [3]
Glycine	0.06	2.36	0.09	2.51	1.0 [3]; 1.8 [3]
Cystine [6]	0.016	0.010	0.008	0.034	0.4 [3, 2]

[1] The amount of tryptophane and methionine in the oxidized casein was determined in this laboratory; all other values were taken from either Toennies ('42), Block and Bolling ('45), Stokes et al. ('45), or Lassen and Bacon ('46).

[2] Almquist and Grau ('44).

[3] Cravens, Almquist, Norris, Bethke and Titus ('44).

[4] Grau and Peterson ('46).

[5] Grau and Almquist ('44).

[6] Not an essential amino acid.

TABLE 2

Comparison of the growth promoting properties of several rations.

CHICKS USED	WHITE LEGHORN [1]			NEW HAMPSHIRE × WHITE LEGHORN [1]		
Basal diet	*dl*-tryptophane added	Number chicks	Gain per chick	*dl*-tryptophane added	Number chicks	Gain per chick
	%		*gm*	%		*gm*
12% oxidized casein	0.8	10	15	0.6	10	144
12% casein	0.5 [2]	8	19	0.34 [2]	10	149
18% oxidized casein	0.8	9	15			
18% casein	0.45 [2]	8	16			
Practical chick starter		8	16		10	148

[1] Day-old White Leghorn chicks were fed the experimental diets for 8 days. The crossbred chicks were placed on experiment at 10 days of age. Experiment conducted for 14 days.

[2] The *l*(-)tryptophane contents of the casein diets were adjusted to approximately equal those of the oxidized diets.

basis for the second method for the determination of the tryptophane requirement of the chick. The technique used in this method involved the substitution of casein for oxidized casein in the basal ration and the feeding of graded levels of casein as the source of tryptophane. The casein added in excess of 12% was supplied at the expense of the starch.

Since the tryptophane requirement of the chick is influenced by the amount of nicotinic acid in the ration, 5 and 10 mg of nicotinic acid per 100 gm of ration were fed with a suboptimum level of tryptophane. The growth response was the same for both groups thereby indicating that, for the ration used, 5 mg nicotinic acid per 100 gm of ration supplied an adequate quantity of this dietary constituent, and this level of nicotinic acid was used in all subsequent experiments.

Studies with graded levels of tryptophane. It had been previously reported that the requirement for tryptophane in the *dl* form was twice that for the natural form, and that the chick required approximately 0.25% l(-)tryptophane in the diet (Grau and Almquist, '44). Therefore the first experiment in this series was designed to feed graded levels of *dl*-tryptophane [3] ranging above and below the recommended level. The crossbred chicks were used in this experiment. The chicks receiving the basal diet to which no tryptophane had been added lost 18 gm (table 3). Approximately 70% of this loss in weight occurred during the first 5 days. The deficient chicks were less active and they remained inside the heated portion of the brooder most of the time. The primary wing feathers were ragged, comb growth was retarded and the chicks appeared weak and emaciated. Typical birds fed the tryptophane deficient and tryptophane supplemented ration are shown in figure 1.

An analysis of variance showed no significant difference in growth between groups fed either 0.30, 0.40, 0.50, or 0.60% *dl*-tryptophane. The chicks fed these diets gained 9 to 10 gm per day, which is considered excellent growth, and the maxi-

[3] We are indebted to The Dow Chemical Company for generous gifts of *dl*-tryptophane.

TABLE 3

Effect of feeding graded levels of tryptophane and casein on growth and efficiency of gain.

	NUMBER CHICKS	WEIGHT AT 24 DAYS	GAIN PER CHICK [1]	GAIN PER GM FEED CONSUMED
		gm.	gm.	gm.
Studies with tryptophane (New Hampshire × White Leghorn chicks)				
Tryptophane added to basal ration (%)				
Experiment 1				
None	10	76	18	— 0.41
0.30 *dl*	10	219	124	0.42
0.40 *dl*	10	229	135	0.47
0.50 *dl*	10	224	130	0.46
0.60 *dl*	10	238	144	0.48
Experiment 2				
None	10	66	— 17	
0.10 *l*(-)	10	102	20	0.13
0.15 *l*(-)	10	194	112	0.38
0.20 *l*(-)	10	223	141	0.49
0.30 *dl*	10	225	143	0.50
0.40 *dl*	10	221	139	0.46
Experiment 3				
0.125 *l*(-)	10	108	27	0.15
0.175 *l*(-)	10	213	133	0.48
0.250 *l*(-)	10	204	123	0.43
0.30 *dl*	10	201	121	0.44
0.50 *dl*	10	208	128	0.44
Casein added to basal ration (%)		**Studies with casein**		
		New Hampshire × White Leghorn		
12	10	191	110	0.44
15	10	231	151	0.54
18	10	235	154	0.56
		White Leghorn		
12	10	182 [2]	92 [2]	0.42
15	10	186	96	0.48
18	10	200	110	0.49

[1] Chicks were fed the experimental diet for 14 days.

[2] Average weight at 22 days.

[3] Chicks were fed the experimental diet for 12 days.

mal efficiency of gain obtained was 0.48. In terms of the reported tryptophane requirement, this was interpreted to mean either that the reported requirement was too high or that the chick was capable of utilizing the *d*-isomer of tryptophane or both. With the use of these experimental techniques, detailed

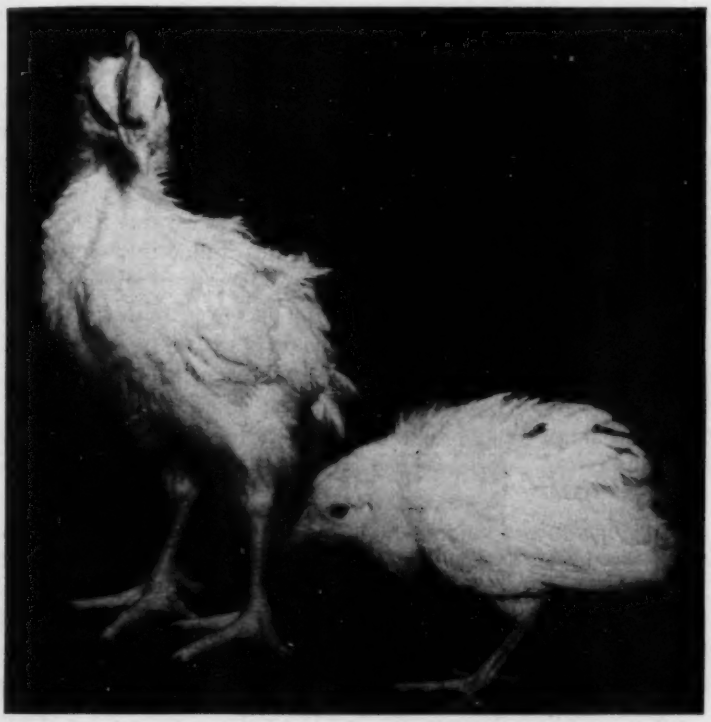

Fig. 1 New Hampshire × White Leghorn chicks after 14 days on experiment. The chick on the left received an adequate diet and weighed 222 gm, while the chick on the right received the basal diet and weighed 63 gm.

data have been obtained on the utilization of the *d*-isomer of tryptophane by the chick and the utilization observed ranged from 17 to 40% (Wilkening and Schweigert, '47).

Experiment 1 indicated that 0.30% *dl*-tryptophane may supply adequate tryptophane to meet the chicks' requirement,

therefore experiment 2 was designed to expand and further investigate the findings of the first experiment. Crossbred chicks were fed graded levels of *l*(-)tryptophane ranging from none to 0.20%. Diets containing 0.30 and 0.40% *dl*-tryptophane were fed as the positive controls. The results of growth and efficiency of gain for the second experiment are presented in table 3.

The ration which contained 0.20 *l*(-)tryptophane supported growth comparable to that obtained with rations supplemented with 0.30 or 0.40% *dl*-tryptophane. It was obvious that the rations containing 0.0 and 0.10% *l*(-)tryptophane did not support growth comparable to rations containing the higher levels of tryptophane. A statistical analysis of the data showed that there was no significant difference in the growth of chicks fed diets containing 0.20% *l*(-)tryptophane and 0.30 and 0.40% *dl*-tryptophane. The gains observed with the 0.15% *l*(-)tryptophane ration were significantly different from those obtained with the 0.20% *l*(-)tryptophane ration ($P < .02$). Thus it was shown that 0.20% *l*(-)tryptophane and 0.30 and 0.40% *dl*-tryptophane supplied an adequate quantity of tryptophane to support both optimum gains and efficiency of gain.

A third experiment was then conducted to further extend and confirm the results obtained in the first 2 experiments. Rations containing 0.125, 0.175, and 0.250% *l*(-)tryptophane and 0.30 and 0.50% *dl*-tryptophane were fed to the crossbred chicks. The results of this experiment are also shown in table 3. In the second experiment it was shown that the 0.15% *l*(-)tryptophane diet did not support maximal growth or efficiency of gain, but the 0.20% diet did. In this experiment, it can be seen that the 0.175% *l*(-)tryptophane diet supplied sufficient tryptophane to the basal ration for optimum growth or efficiency of gain. As was true in the previous experiments for rations supplemented with *dl*-tryptophane, the 0.30% diet supported maximal gains and efficiency of gain.

The growth data obtained with rations containing 0.175 and 0.25% *l*(-) tryptophane and 0.30 and 0.50% *dl*-tryptophane

were treated statistically. The F test showed no significant difference between any of these levels of l(-) or *dl*-tryptophane. Therefore, it was concluded that maximal rates of gain were obtained with diets containing either 0.175% *l*(-) tryptophane or 0.30% *dl*-tryptophane and that higher levels of tryptophane did not result in an increased growth response.

For maximal gain the minimum amount of *l*(-)tryptophane which was added to the basal ration was 0.175%. The basal

TABLE 4

Effect of feeding graded levels of tryptophane on growth and efficiency of gain (White Leghorn).

TRYPTOPHANE ADDED TO BASAL RATION	NUMBER CHICKS	WEIGHT AT 22 DAYS	GAIN PER CHICK [1]	GAIN PER GM FEED CONSUMED
%		*gm.*	*gm.*	*gm.*
None	10	77	—12	—0.14
0.100 *l*(-)	10	107	18	0.15
0.135 *l*(-)	10	156	66	0.33
0.175 *l*(-)	10	178	88	0.44
0.200 *l*(-)	10	181	91	0.38
0.225 *l*(-)	10	184	94	0.44
0.30 *dl*	10	179	89	0.42

[1] The experiments were conducted for 12 days.

ration contained 0.008% *l*(-)tryptophane (table 1). Therefore the minimum level of *l*(-)tryptophane in a diet which supported optimum growth was 0.183%. From the results obtained with the oxidized casein diets, the *l*(-)tryptophane requirement of the New Hampshire × White Leghorn cross was therefore shown to be 0.18% of the diet.

Since the results obtained in this laboratory with respect to the tryptophane requirement of the chick were not in agreement with those reported by Grau and Almquist, whose experiments were conducted with the White Leghorn chick, it appeared desirable to obtain information on the White Leghorn. These workers used a 12-day feeding period, and the experiments performed in this laboratory with the Leghorn were conducted for a similar period of time. The results of this experiment are presented in table 4. While the rate of

gain was less for the White Leghorn, the relative response of the Leghorn to the graded levels of tryptophane was the same as that obtained with the hybrid chick. The growths obtained with rations containing 0.175, 0.20, and 0.225% *l*(-) tryptophane and 0.30% *dl*-tryptophane were all of the same order of magnitude, but the ingestion of rations containing lower amounts of tryptophane resulted in correspondingly less growth. There was no statistically significant difference in the gains obtained with rations containing 0.175, 0.20, and 0.225% *l*(-)tryptophane and 0.30% *dl*-tryptophane. These results are in agreement with those obtained with the crossbred chicks and thereby confirm the requirement of the chick for tryptophane at 0.18% of the ration.

Comparative blood studies were made on normal and deficient hybrid chicks from Experiment 2 which had been on the test for 14 days. In this study determinations were made on the hemoglobin and plasma protein levels and the apparent free tryptophane content of the plasma. The results obtained for hemoglobin and total plasma protein showed no difference between the normal and deficient groups. It appears, therefore, that a longer time would be required for a significant reduction to occur in these blood constituents. However, the apparent free tryptophane levels, determined microbiologically (Schweigert et al., '46) varied with the intake of tryptophane. The average content of apparent free tryptophane in the plasma of the group receiving the basal ration was 1.8 μg per ml, while the average level for the group receiving an adequate amount of tryptophane was 6.5 μg per ml of plasma. This observation confirms previous findings with the rat (Schweigert et al., '46).

Studies with graded levels of casein. Since the preliminary experiments indicated that diets containing untreated casein afforded a second method for determining the tryptophane requirement of the chick, an experiment was conducted using diets in which casein [4] replaced oxidized casein in the basal

[4] The casein used in these experiments analyzed 14.3% total nitrogen, 10.2% moisture, and 1.14 *l*(-)tryptophane. The values for total nitrogen and *l*(-)tryptophane are uncorrected.

ration. The casein was essentially the sole source of tryptophane in the diet, and the tryptophane content of the ration was varied by feeding graded levels of casein. The New Hampshire × White Leghorn cross and the White Leghorn chicks were used in this experiment. The results are presented in table 3. In the experiment with the hybrid chick, the 12% casein diet produced neither optimum gains nor efficiency of gain. It was apparent that both the 15 and 18% casein diets produced a similar response, and statistically there was no significant difference between these groups. The difference in the growth obtained between the 12% and 15% casein diets was highly significant ($P = 0.0012$).

The growth of the White Leghorn on the 15% casein diet was not comparable with that of the group fed the 18% casein ration, but the efficiency of gain was approximately the same for both groups. The group fed 15% casein consumed approximately 215 gm less feed than did either the group fed 12% or 18% casein. There was no available explanation for this decrease in feed consumption. Since the efficiency of gain had reached a maximum for both the White Leghorn and the hybrid and growth was at a maximum for the latter it was concluded that 15% casein supplied a quantity of tryptophane sufficient to meet the chicks' requirement.

The tryptophane content of the casein was determined by microbiological analysis using *Streptococcus faecalis* R as the test organism. The tryptophane was liberated from the casein by alkaline hydrolysis (Kuiken and Lyman, '47). The basal medium used for the determination of the tryptophane was the same as that used by Greenhut, Schweigert and Elvehjem ('46). The *l*(-)tryptophane value obtained for the casein was 1.14%.

It was demonstrated that the 15% casein diet supplied an adequate quantity of tryptophane to meet the chick's requirement and that the casein contained 1.14% *l*(-)tryptophane. Therefore, the 15% casein supplied 0.171% *l*(-)tryptophane while the remainder of the ration supplied 0.005% *l*(-)tryptophane. The total amount of *l*(-)tryptophane contributed by

this ration was 0.176%. Consequently, the tryptophane requirement of the chick as determined with the casein diets was 0.18% of the diet.

The results obtained with the oxidized casein and casein diets are in excellent agreement. The *l*(-)tryptophane requirement of the chick, as determined by 2 independent methods with both the New Hampshire × White Leghorn cross and the White Leghorn, is 0.18% of the diet for either growth or for efficiency of feed utilization.

There is no obvious explanation for the difference in the results obtained in this laboratory and those reported by Grau and Almquist. However, the level of nicotinic acid or other dietary components used may have been contributing factors.

SUMMARY

Chicks were fed a purified, tryptophane-low diet in which the major sources of protein were 12% oxidized casein and 10% gelatin supplemented with cystine and methionine. Chicks grew normally when an adequate amount of tryptophane was added to this diet. Using this ration with graded levels of tryptophane and a ration in which untreated casein supplied the tryptophane, the *l*(-)tryptophane requirement of the New Hampshire × White Leghorn crossbred chick and the White Leghorn chick was shown to be 0.18% of the diet.

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NUTRITIONAL STUDIES ON MILK FAT

III. THE EFFECT OF THE TREATMENT OF MILK FAT WITH CERTAIN SOLVENTS ON THE GROWTH OF YOUNG RATS

E. L. JACK AND E. B. HINSHAW
Division of Dairy Industry, University of California, Davis

FIVE FIGURES

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Previous experiments (Jack et al., '45) have shown that young rats fed diets containing milk fat which had previously been dissolved in pentane (Skelly-solve A) did not grow as well as those fed diets containing untreated milk fat. Kratzer ('45) noted that the use of chloroform as a solvent had a similar effect on chicks. Examination of the fat which had been dissolved in pentane showed that it was not oxidized by the treatment, but was less resistant to oxidation as measured by the development of peroxides in accelerated oxidative stability tests at 80°C.

It seemed probable that the retardation of growth could have been caused by a destabilizing effect of the solvent either through chemical reaction or by a physical reorientation of the natural antioxidants present. The possibility of an impurity in the solvent which could have caused retardation of growth was not overlooked, although this was not considered likely since the solvent had a negative peroxide test.

EXPERIMENTAL

A series of diets containing milk fat which had been dissolved in various solvents, followed by solvent-removal before feeding, was prepared and fed as described earlier (Hender-

son et al., '45). Two male and 2 female rats weighing about 45 gm at weaning were used in each lot. The solvents chosen were methyl alcohol, ethyl ether, acetone, chloroform, and pentane as representing the most common types of compounds usually used as fat solvents. The solvent was removed by distillation and subsequent sweeping with nitrogen to remove the last traces.

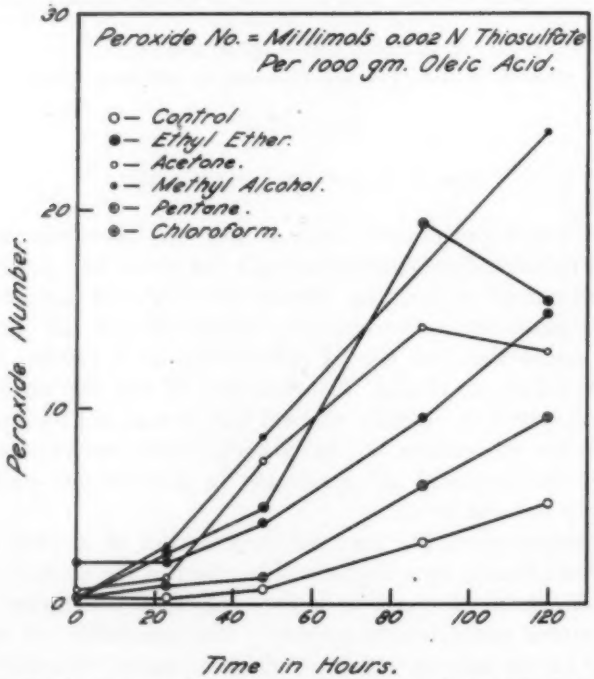

Fig. 1 The development of peroxides in milk fat treated with different solvents.

Figure 1 shows the oxidative stability of the treated fats. The acetone and chloroform were redistilled and ethyl ether was freed from peroxides over metallic sodium. Pentane does not show as great a destabilizing effect as the other solvents, but it should be essentially inert since it is a saturated hydrocarbon.

Figure 2 shows the growth of rats fed diets containing 20% milk fat treated with the previously mentioned solvents. This is a representative trial among several that were run, all showing essentially the same relationships. Pentane retarded

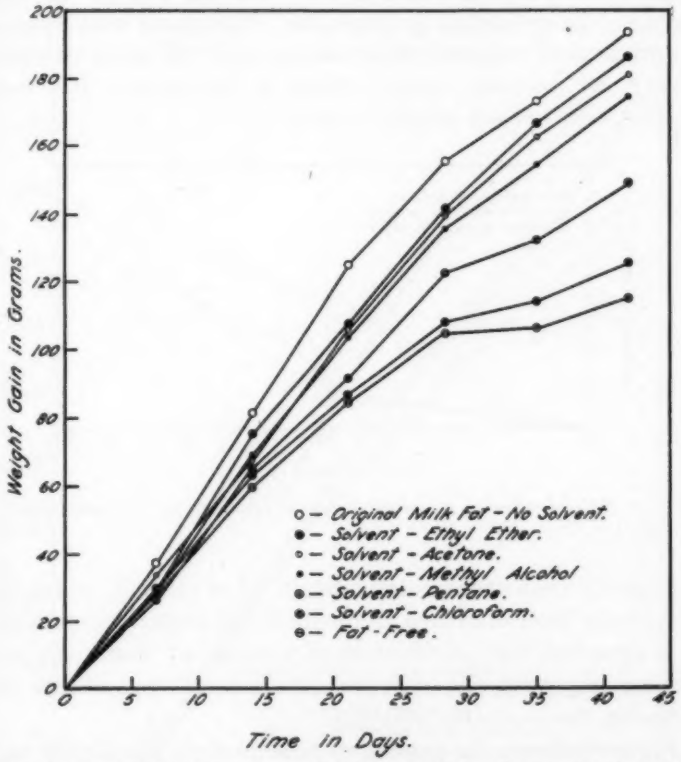

Fig. 2 The growth of young rats fed diets containing fats previously treated with different solvents.

growth to a greater degree than it reduced the oxidative stability and this led to an examination of the solvent for possible unsuspected impurities. Methyl alcohol caused the greatest increase in peroxide number but depressed growth very little. Ethyl ether did not depress growth but produced a high

peroxide number. There was, therefore, very little correlation between growth and peroxide number.

Sulphur is a common impurity in solvents of petroleum derivation but no evidence of sulphur could be found. A slight gummy residue that developed upon drying was suspected of being olefinic in character. Treatment with fuming sulphuric acid removed this residue and following this discovery, all pentane, except where so designated, has been treated with fuming sulphuric acid.

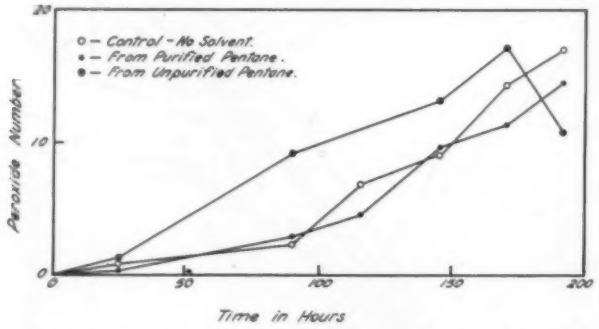

Fig. 3 The development of peroxides in milk fat as affected by purification of pentane.

Figure 3 shows the oxidative stability of milk fat which has previously been dissolved in purified and unpurified pentane. It is apparent that purification of pentane by treatment with fuming sulphuric acid removed the material responsible for reducing the oxidative stability.

Figure 4 shows the growth of rats on diets containing milk fat that had been dissolved in purified and unpurified pentane and also the growth on diets containing 3 milk fat fractions from purified pentane, prepared as described by Henderson and Jack ('44). Apparently, the deleterious effects of the impurities were removed since the fat which had been dissolved in the purified pentane produced the same growth as that which had not been dissolved in solvent.

In the previous experiments (Jack et al., '45) it was reported that milk fat, not solvent treated, produced slightly greater growth than the —53° filtrate fraction. It will be noted that the —53° filtrate used here from purified pentane produced growth substantially greater than the original fat.

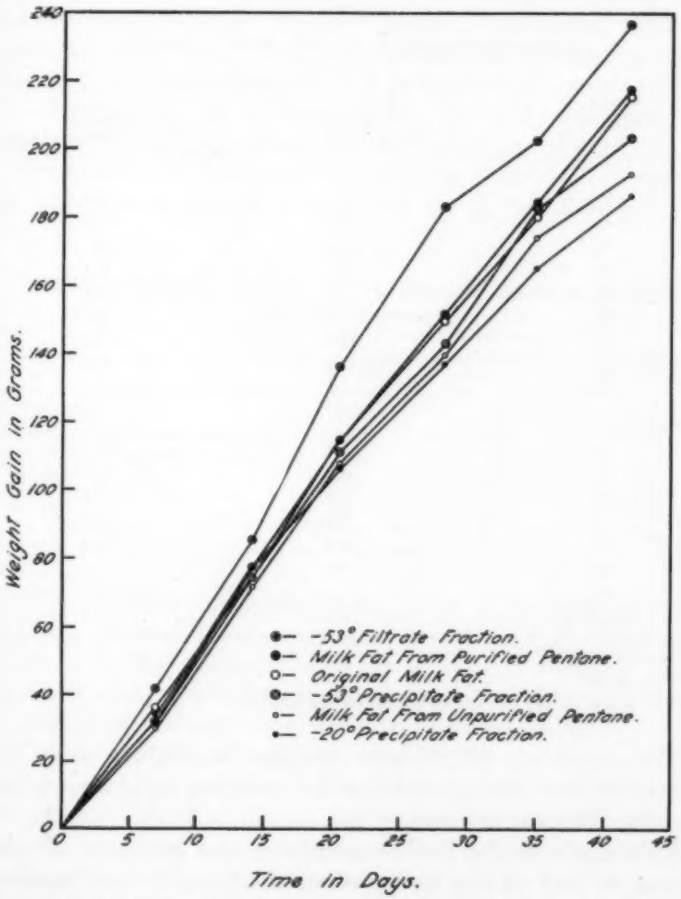

Fig. 4 The growth of young rats fed diets containing fat from purified and unpurified pentane.

This was to be expected since the —53° filtrate used previously was prepared with unpurified pentane.

Table 1 gives the fatty acid composition of the —53° filtrate fraction used in this experiment, together with the composition of that used previously for comparison.

TABLE 1

Fatty acid composition of —53° filtrate fractions.

LENGTH OF CARBON CHAIN	MOL PER CENT	
	From unpurified pentane [1] I.V.[3] 58.37, Sap. no. 236.9	From purified pentane [2] I.V. 60.8, Sap. no. 235.4
Saturated		
C_4	9.3	4.2
C_8	6.9	1.6
C_8	2.4	3.7
C_{10}	4.5	1.1
C_{12}	3.7	2.4
C_{14}	7.4	5.4
C_{16}	14.0	11.8
C_{18}	6.8	9.2
C_{20}	1.4	..
Unsaturated		
C_{10}	1.4	0.3
C_{12}	0.4	1.1
C_{14}	1.8	1.1
C_{16}	4.6	11.7
C_{18}	30.0	32.4
C_{20}	0.8	4.9
C_{22}	..	0.7
Linoleic	6.0	8.4

[1] Jack et al. ('45).

[2] This study.

[3] I.V. is iodine value; Sap. is saponification number.

The present —53° filtrate fraction is slightly more unsaturated than the one previously used but otherwise is not greatly different in composition.

Table 2 shows the feed consumption and efficiency of utilization of feed on the diets containing the milk fat fractions prepared from purified pentane. These values are for the first 5 weeks only. The values for the entire 6 weeks for all diets

are not available because of failure of the attendant to weigh the remaining feed for some of the animals at the end of the trial. The data available indicate that there is no significant difference in 6 weeks from the 5-weeks' results.

It will be noted that the total feed consumption is not greatly different on the different diets. This is in accord with the previous findings by Jack et al. ('45) when milk-fat fractions were fed. The animals on the diet containing —53° filtrate fraction ate the least feed and gained the most weight. The animals on the diet containing the —20° ppt. fraction required the largest amount of feed per gram of gain.

The reports by Burr and his associates ('43, '44) concerning the deleterious effects of oxidized fat in the diet and the

TABLE 2

Feed consumption and efficiency of feed utilization on milk fat fractions from purified pentane.

DIET	FEED CONSUMED 5 WEEKS	GAIN IN WEIGHT 5 WEEKS	GRAMS FEED PER GRAM OF GAIN
Original fat	449	181	2.49
Original fat (Purified pentane)	444	184	2.41
Original fat (Unpurified pentane)	467	175	2.67
—20° ppt.	474	166	2.85
—53° ppt.	488	183	2.67
—53° Filtrate	441	203	2.18

stabilizing effects of tocopherols, taken in conjunction with the data presented here, suggested a possible nutritional significance in the oxidative stability of fat. A series of diets was prepared in which milk fat was used at different levels of oxidative stability as altered in the laboratory.

Milk fat unoxidized and with no peroxide number was used in the control. A portion of this fat was carried to the end of its natural induction period, to a peroxide number of 1–2. Another portion was mildly oxidized by bubbling oxygen through it to a peroxide number of 10–12. And a fourth sample was fortified with 0.15% of mixed tocopherols after being taken to the end of its natural induction period. These

fats were mixed in diets as before at a level of 20% and fed to young rats. These results are shown in figure 5.

The growth differences are small; however, the mildly oxidized fat did not produce quite as good growth as the other samples containing fat. Neither reducing the induction period nor fortifying the destabilized fat with tocopherol had any significant effect on growth.

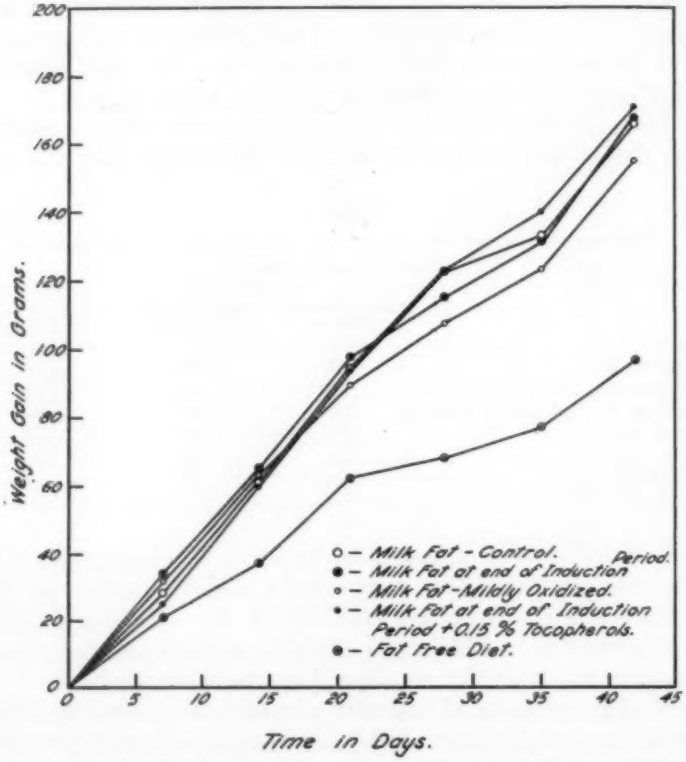

Fig. 5 The growth of young rats on diets containing milk fat in different stages of oxidation.

DISCUSSION OF RESULTS

The growth-retarding effects of solvents on fats to be used in experimental diets evidently result from a residue of a

deleterious component, where the fat has not interacted chemically with the solvent, and where the fat has not become oxidized. Reduction of the oxidative stability, short of actual oxidation, in these experiments, did not affect the growth of young rats when fed diets containing milk fat treated thusly. Also, mildly oxidized fat did not reduce growth to the same extent as the unpurified pentane.

Noteworthy is the significantly greater growth resulting from the diet containing the —53° filtrate milk-fat fraction prepared with purified pentane. The animals on this diet were able also to use their feed more efficiently. It seems probable that a growth-promoting component from milk fat is concentrated in this fraction. Studies are under way to identify this possible component and to determine its mode of action.

The authors do not have any satisfactory explanation to account for the differences in weight gains as represented by the same diets in figures 2, 4 and 5.

SUMMARY AND CONCLUSIONS

1. Experiments have shown that pentane (Skelly-Solve A) contains an impurity which lowers the growth-promoting value of milk fat. The oxidative stability is also lowered.

2. Purification of the solvent by treating with fuming sulphuric acid removes this deleterious component.

3. Fat dissolved in purified pentane is not reduced in oxidative stability nor in growth-promoting value.

4. The —53° filtrate milk-fat fraction prepared with purified pentane produced significantly greater growth in young rats than the original milk fat and also greater than the other milk fat fractions.

5. Young rats fed a diet containing —53° filtrate milk-fat fraction used their feed more efficiently for growth than those fed diets containing the original milk fat and the other fractions.

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AMINO ACID DEFICIENCIES OF RAW AND OVERHEATED SOYBEAN OIL MEAL FOR CHICKS[1]

JAMES McGINNIS AND ROBERT JOHN EVANS[2]
Divisions of Poultry Husbandry and Chemistry, Washington Agricultural Experiment Station, Pullman, Washington

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Morgan ('31) found that the biological value for rats of cereal proteins and casein was decreased by dry heating at 140°C. for 30 minutes. Later it was shown (Greaves, Morgan and Loveen, '38) that lysine was very effective in correcting the amino acid deficiency of heated casein. Boiling or autoclaving of meat protein (Morgan and Kern, '34) and autoclaving of edestin (Waisman and Elvehjem, '38) for 5 hours at 120°C. reduced the biological value of these proteins for rats. A supplement of lysine to the autoclaved edestin gave better growth than did the unautoclaved material. Overheating of cereal and other proteins in the explosion process used in the preparation of many breakfast foods caused a marked decrease in protein nutritive value (Stewart, Hensley and Peters, '43; Mitchell, Hamilton and Beadles, '45).

The nutritive value of soybean oil meal was shown to be improved by proper heat treatment and impaired by autoclaving for too long a time or at too high a temperature (Parsons et al., '39; Bird and Burkhardt, '43; Evans and McGinnis, '46).

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[2] Present address, Division of Chemistry, Michigan State College, East Lansing, Michigan.

Parsons et al. ('39) reported that lysine failed to improve the nutritive value of over-heated soybean oil meal. Evans and McGinnis ('46), using a diet containing 5% dried brewers' yeast and 5% gelatin in addition to the soybean oil meal, found that methionine added to the diet containing over-heated soybean oil meal (autoclaved for 30 minutes at 130°C.) gave a chick growth response almost equal to that obtained with optimally heated soybean oil meal plus methionine.

Clandinin et al. ('47) found that over-heated soybean oil meal, when incorporated in a practical soybean oil meal chick diet, was deficient in available methionine and lysine. Since in the earlier work (Evans and McGinnis, '46; Clandinin et al., '47) all of the dietary protein was not supplied by soybean oil meal, it was desirable to determine whether amino acids in soybean oil meal other than methionine and lysine are made unavailable to the chick by prolonged autoclaving.

EXPERIMENTAL

The raw soybean oil meal used in this study was a solvent-extracted product. The required amount for each diet was spread in enamel pans to a depth of about 1 inch and then autoclaved. As indicated in table 1, some samples were autoclaved for 30 minutes at 100°C., whereas, others were autoclaved for 60 minutes at 130°C. These treatments had previously been found (Evans and McGinnis, '46) to give a soybean oil meal with high and low nutritive value, respectively.

The percentage composition of the diets fed was as follows: Soybean oil meal 48.0, cerelose 43.9, mineral mixture [3] 5.0, soybean oil 2.5, fish oil (400 A.O.A.C. units D, 1000 U.S.P. units A/gm) 0.5, ethanol-soluble liver fraction 0.1. Vitamins were added in milligrams to each 100 gm of diet as follows: choline chloride 200, mixed tocopherol concentrate 10.0, p-aminobenzoic acid 10.0, niacin 3.0, 2-methyl 1, 4-naphtho-

[3] Composition of mineral mixture (grams): Ground limestone 1570, dicalcium phosphate 1740, K_2HPO_4 840, NaCl 600, $MgSO_4 \cdot 7\,H_2O$ 500, $Fe_2(SO_4)_3 XH_2O$ 55, $MnSO_4 \cdot 4\,H_2O$ 29, KI 3.3, $CuSO_4 \cdot 5\,H_2O$ 1.5, $ZnCl_2$ 1.0, $CoCl_2 \cdot 6\,H_2O$ 0.2.

quinone 1.0, riboflavin 1.0, calcium pantothenate 1.0, thiamine 0.5, pyridoxine HCl 0.5, pteroylglutamic acid 0.05, biotin 0.01. The diets contained 22.4% protein, 0.26% methionine and 0.46% cystine. The methionine and cystine were determined by the differential oxidation procedure (Evans, '45). The only differences between the various diets were the different autoclaving treatments that the raw soybean oil meal received and the amino acid supplements which are described in table 1. The amount of cystine and methionine added to the diets was sufficient to meet the chick's requirement for these 2 amino acids, according to Grau and Almquist ('43).

Unsexed New Hampshire chicks were distributed at random into 30 groups of 10 chicks each. The chicks were wing banded and placed in electrically heated battery brooders. Each experimental diet and water were fed *ad libitum* to duplicate groups of chicks. The chicks were weighed individually and feed consumption determined at weekly intervals. The duration of the experiment was 4 weeks.

RESULTS

The results on chick growth and feed utilization are summarized in table 1. The unautoclaved raw soybean oil meal gave poor growth, and a supplement of cystine failed to appreciably improve the growth of chicks fed the raw soybean oil meal. Methionine gave a much greater growth response than cystine but failed to give maximum growth. A combination of methionine and cystine gave about the same growth as methionine alone.

Autoclaving the raw soybean oil meal for 30 minutes at 100°C. gave a pronounced growth response which was more than twice as great as that given by the supplement of methionine to the raw soybean oil meal. Growth of chicks fed diets containing soybean oil meal autoclaved for 30 minutes at 100°C. was not improved by supplements of either (1) methionine alone, (2) cystine alone, (3) methionine and cystine, or (4) methionine, cystine and lysine. These results together with the very satisfactory growth results at 4 weeks indicate

TABLE 1

The influence of amino acid supplements on the nutritive value of soybean oil meal given different autoclaving treatments.

AUTOCLAVING OF RAW SOYBEAN OIL MEAL		ADDITIONS TO DIET	AVERAGE WEIGHT AT 4 WEEKS		PROTEIN EFFICIENCY
Time = min. Temp. = °C.		*gm/100 gm*	*gm*	*gm*	*(gain per gm protein)*
None		None	107 138	123 [1]	1.4
None		0.5 cystine	131 143	137	1.5
None		0.5 methionine	207 184	196	1.8
None		0.5 methionine 0.5 cystine	156 212	186	1.6
30	100	None	325 287	306	2.1
30	100	0.5 cystine	319 324	322	2.3
30	100	0.5 methionine	284 288	286	2.2
30	100	0.5 cystine 0.5 methionine	304 300	302	2.2
30	100	0.5 methionine 0.5 cystine 0.5 lysine	295 301	298	2.3
60	130	None	161 198	180	1.5
60	130	0.5 cystine	164 169	167	1.5
60	130	0.5 methionine	166 188	177	1.5
60	130	0.5 cystine 0.5 methionine	213 186	201	1.6
60	130	0.5 lysine	157 176	167	1.5
60	130	0.5 cystine 0.5 methionine 0.5 lysine	290 318	304	2.0

[1] Average of duplicates.

that 48% of soybean oil meal in the diet meets the chick's need for amino acids and also that autoclaving at 100°C. for 30 minutes did not destroy or render unavailable an appreciable amount of methionine, cystine or lysine.

In contrast to the excellent growth results obtained with the soybean oil meal autoclaved at 100°C. for 30 minutes, chicks grew poorly when fed soybean oil meal autoclaved for 60 minutes at 130°C. This treatment had previously been found to decrease the retention of methionine and cystine by chicks (Evans and McGinnis, '46). In the earlier work in which soybean oil meal supplied about 60% of the dietary protein, a supplement of methionine to soybean oil meal autoclaved 30 minutes at 130°C. gave maximum growth. The results in table 1 show that supplementary methionine, cystine and lysine when added singly to the diet containing the overcooked soybean oil meal failed to improve growth. A combination of methionine and cystine did not correct the heat damage to the soybean oil meal protein. On the other hand, a combination of methionine, cystine and lysine when added to the diet containing overcooked soybean oil meal corrected the amino acid deficiencies of the heat-damaged soybean oil meal. Since a combination of cystine and lysine or of methionine and lysine was not used, it cannot be determined from the data whether methionine or cystine was destroyed or made unavailable by autoclaving soybean oil meal at 130°C. for 60 minutes.

DISCUSSION

The greater growth response obtained in this work as compared to that obtained earlier (Evans and McGinnis, '46) by autoclaving for 30 minutes at 100°C. is probably explained by the difference in the amount of dietary protein supplied by soybean oil meal. The diets used in the work reported in this paper contained all of the protein from soybean oil meal, whereas, the diets used in the earlier work (Evans and McGinnis, '46) had about 40% of the protein supplied by dried brewers' yeast and gelatin. Both of these materials are poor sources of methionine and cystine. Under the conditions of the

experiment reported in this paper, methionine was not a growth-limiting deficiency in the diets containing soybean oil meal autoclaved for 30 minutes at 100°C. On the other hand, according to Grau and Almquist ('43), the 0.26% of methionine and 0.46% cystine supplied by 48% of soybean oil meal would not be sufficient to meet the chick's requirement for these amino acids. Grau and Almquist ('43) reported that the chick needs about 0.55% methionine and a total of 1.0–1.1% of methionine and cystine for maximum growth.

The effect of methionine in correcting the heat damage to soybean oil meal caused by autoclaving for 30 minutes at 130°C. in the earlier work (Evans and McGinnis, '46) was probably due to the lysine supplied by the dried brewers' yeast and gelatin and to less destruction of lysine. The failure of lysine to correct the heat damage to soybean oil meal reported by Parsons et al. ('39) may have been due to a critical deficiency of methionine.

The decreased liberation of nitrogen from soybean oil meal by *in vitro* enzymatic digestion with pepsin, trypsin, and erepsin caused by overcooking (Evans, '46) suggests that the biological value might be impaired by rendering of the proteins indigestible. The data obtained with the chick by supplementing the heat-damaged soybean oil meal with methionine, cystine and lysine indicate that these particular amino acids rather than the entire proteins were affected by overcooking under the experimental conditions.

SUMMARY

Data obtained with chicks using a diet in which soybean oil meal supplied all of the protein permit the following conclusions regarding the effect of autoclaving on the biological value of soybean oil meal proteins:

1. Chick growth was not improved by supplementing a diet containing raw soybean oil meal with cystine. The addition of methionine to this diet gave a growth response that was not maximum.

2. Autoclaving raw soybean oil meal at 100°C. for 30 minutes gave a marked increase in its nutritive value as shown by chick growth. Neither methionine nor cystine nor a combination of these 2 amino acids improved chick growth when added to the diet containing soybean oil meal autoclaved at 100°C. for 30 minutes. Furthermore, a combination of cystine, methionine, and lysine failed to improve growth.

3. The nutritive value of soybean oil meal was decreased by autoclaving at 130°C. for 60 minutes. The heat damage to the soybean proteins caused by this treatment was not corrected by the addition of methionine, cystine, or lysine. A combination of these 3 amino acids corrected the heat damage caused by this autoclaving treatment.

4. The growth response given by autoclaving raw soybean oil meal at 100°C. for 30 minutes was more than twice as great as the response given by a supplement of methionine. This indicates that autoclaving raw soybean oil meal improves the nutritive value by affecting the availability of nutrients other than cystine or methionine.

5. The methionine requirement of the chick appeared to be not more than 0.26% when the diet contained 0.46% cystine. It may be possible that New Hampshire chicks require less methionine than White Leghorns.

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RUMEN DIGESTION STUDIES

I. A METHOD OF INVESTIGATING CHEMICAL CHANGES IN THE RUMEN [1]

E. B. HALE,[2] C. W. DUNCAN AND C. F. HUFFMAN

Departments of Dairy and Agricultural Chemistry, and the Institute of Nutrition, Michigan State College, East Lansing, Michigan

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A universal practice among investigators of ruminant nutrition is one of frequent reference to the importance of the rumen in accomplishing the disintegration of plant materials. Yet, amazingly little is known of the nature and extent of such disintegration, of the responsible organisms, or of the relation of rumen action to subsequent action in other divisions of the digestive tract. Consequently, a comprehensive concept of rumen digestion has not been possible. Progress in the elucidation of the chemical changes taking place in the rumen has been slow due to a lack of appropriate experimental techniques. In early studies with fistulated animals results were interpreted in terms of the percentage composition of the rumen contents as compared with the hay fed (Krzywanek and Quittek, '36) or by comparing the composition of the rumen contents as digestion progressed (Silver, '35). Burroughs, Gerlaugh, Silver and Schalk ('46) reported a chemical method for identifying individual feeds within the rumen of cattle receiving a mixed ration containing corn, hay and a protein supplement. This method was based upon a mathematical treatment of the principle nutrients contained in the ingested feeds and the nutrients present in the rumen ingesta.

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[2] Present address, University of Chicago, Chicago, Illinois.

Studies of this nature only yield data of relative value and give little indication of the actual changes. Hale, Duncan and Huffman ('40), however, pointed out the application of lignin ratios to investigations of rumen digestion, and McAnally ('42) reported on the value of suspending silk sacs containing ground straw in the rumen. These methods seem to circumvent many of the complications introduced by a moving system and promise to yield information of a fundamental nature.

In the present paper a critical appraisal of the lignin ratio method is made and a technique for measuring the rate of rumen digestion is presented. In a previous study (Hale, Duncan and Huffman, '40) lignin was assumed to be indigestible in the rumen, whereas the present communication demonstrates a method of measuring the digestibility of lignin in the rumen.

METHODS

Chemical procedures. Lignin, cellulose and other carbohydrates were determined by the procedures of Crampton and Maynard ('38) and all the other nutrient values were obtained by the A.O.A.C. methods ('40).

Measurement of concentration by means of lignin ratios. In a preceding paper (Hale, Duncan and Huffman, '40) lignin ratios were used to calculate rumen digestion coefficients. This method was based upon the assumption that lignin was not digested to any appreciable extent in the rumen. An assumption of this character permits a measurement of the concentration of the rumen contents by comparing the per cent of lignin in the rumen with that determined in the ingested hay. The method of calculating the rumen coefficients is represented in the following formula:

$$(1) \qquad 100 - \left(\frac{\text{Per cent lignin in hay}}{\text{Per cent nutrient in hay}}\right) \times \left(\frac{\text{Per cent nutrient in rumen}}{\text{Per cent lignin in rumen}}\right) \times 100 = \text{Digestion coefficient of nutrient}$$

The validity of this method is dependent upon the degree to which lignin is attacked in the rumen. Evidence of the indigestibility of lignin was obtained by application of the

method to rumen materials (Hale, Duncan and Huffman, '40). Any digestion of lignin would have resulted in low digestion coefficients, yet calculated rumen coefficients were high and approached or equaled fecal coefficients. Data of this nature suggest that lignin does not undergo any appreciable digestion in the rumen; nevertheless, more specific information regarding this point is necessary. Digestion trials reported by Ellis, Matrone and Maynard ('46) indicate that lignin is not digested by the cow, sheep and rabbit.

To fill the need for *a measurement of the digestibility of lignin in the rumen,* thereby enabling the investigator to correct rumen digestion coefficients for any discrepancies arising from lignin digestion, the following formula may be used:

$$(2)^{3} \quad \frac{\left(\begin{array}{c}\text{Pounds of crude}\\ \text{fiber digested}\end{array} + \begin{array}{c}\text{Pounds of N.F.E.}\\ \text{digested}\end{array}\right) - \left(\begin{array}{c}\text{Pounds of cel-}\\ \text{lulose digested}\end{array} + \begin{array}{c}\text{Pounds of other}\\ \text{CHO digested}\end{array}\right)}{\text{Pounds of lignin in hay}} \times 100 = \text{Digestion coefficient of lignin}$$

This formula is possible since the sum of crude fiber and nitrogen-free extract is identical to the sum of cellulose, other carbohydrates and lignin. Thus any difference in the digestibility of the 2 fractions represented in the formula would be due to the digestibility of lignin. The digestion coefficients derived from formula (1) were obtained originally by assuming lignin to be indigestible. Although any digestion of lignin would result in calculated coefficients lower than the real coefficients, the error would be of the same magnitude for both of the involved fractions. The accuracy of the calculation would not be appreciably impaired because the difference between these 2 fractions would remain essentially unaltered.

An idea of the applicability of formula (2) may be obtained by applying it to fecal material of known digestibility coefficients. By using the data in columns 1 and 7 of table 2 and columns 3 and 8 of table 3 in the accompanying paper Hale, Duncan and Huffman ('47) found the calculated lignin digestion coefficients to be 31.3 and 21.9% as compared with the respective actual values of 32.7 and 21.5% (table 3). These

[3] Pounds of a nutrient refers to the amount per 100 pounds of hay.

values appear to be within the limits of the experimental error encountered in digestion trials.

Corrections for rumen digestion coefficients obtained by formula (1) can readily be made if an appreciable digestion of lignin is encountered in the rumen. In making the calculations, the percentage of indigestible lignin in the hay is used rather than the original lignin content of that hay. For example, if the hay contained 15.0% lignin and if 4% of the lignin was digested in the rumen, then $0.15 - (0.15 \times 0.04) \times 100$ or 14.4 would replace the 15.0% value in the formula for calculating the rumen coefficient. Thus, a formula free of any discrepancies introduced by the possible disintegration of lignin in the rumen is obtained with which rumen digestion coefficients can be calculated:

$$(3) \qquad 100 - \left(\frac{\text{Per cent indigestible lignin in hay}^4}{\text{Per cent nutrient in hay}} \times \frac{\text{Per cent nutrient in rumen}}{\text{Per cent lignin in rumen}}\right) \times 100 = \text{Digestion coefficient of nutrient}$$

In table 3 in the accompanying paper (Hale, Duncan and Huffman, '47) both the original and corrected coefficients of rumen digestion are presented.

Limitations of the lignin ratio method. The lignin ratio method is not adapted to the study of rations containing concentrates and it gives significant values for roughages only after rumen digestion has reached a maximum.

As concentrates are deficient in lignin a suitable lignin value cannot be obtained for measuring their concentration. In a mixed ration differences in the rate of passage of concentrates and fibrous materials would invalidate the calculations. Investigations of the rumen disintegration of concentrates and mixed rations might better be carried out by means of the silk-sac method described by McAnally ('42). Results obtained by this method are limited to comparative interpretations alone since concentrates would be retained in the rumen beyond the period of normal passage and, as McAnally's data for straw suggest, a retarded digestion may occur (3 days required for 40% digestion). The simplicity of this method,

[4] This term refers to indigestibility in the rumen.

however, and the large number of samples that may be studied recommend its application in comparative and preliminary investigations.

The limitation of the lignin ratio method for any measurement other than that of the maximum concentration in the rumen can readily be noted. When an animal consumes the feed placed before it, the freshly ingested material is mixed with the highly concentrated rumen contents of the previous digestion period. This means that a sample collected from the rumen immediately after feeding would indicate, on the basis of lignin ratios which measure concentration, that considerable disintegration had occurred while in reality only a mechanical mixing of the hay and the previously concentrated rumen contents had taken place. If simple lignin ratios are used to calculate the digestibility of dry matter in the rumen at zero hours after feeding on the basis of the data in table 2 in the accompanying paper (Hale, Duncan and Huffman, '47), a value of 38.3% would be obtained. This value represents mechanical concentration only and indicates the magnitude of the error resulting from such concentration. The results obtained by Rathnow ('38) further demonstrate this limitation. He used the simple ratio method, employing iron rather than lignin, for studies of the rate of rumen digestion and obtained values of 32.1, 48.5 and 58.8% for the digestibility of dry matter at 3, 6 and 9 hours, respectively, after ingestion. These values, particularly those for 3 and 6 hours, are unduly high and are of the magnitude that might be expected from mixing the ingesta with the concentrated rumen contents.

As lignin ratios do not differentiate between mechanical concentration and concentration resulting from current disintegrative activity, they are limited to the determination of the maximum concentration occurring in the rumen. For this purpose the method would seem well adapted and subject only to those errors innate in ordinary digestion trials.

Measurement of the passage of nutrients from the rumen or a method for studying the rate of rumen digestion. A moving system such as that encountered in the study of rumen diges-

tion presents complications which must be thoroughly understood before undertaking an investigation of the rates of chemical changes. In the course of a 12-hour digestion period between feedings passage of nutrients from the rumen is effected by 2 distinct processes. These are (a) passage of the concentrated rumen contents remaining in the rumen from previous digestion periods,[5] and (b) separation of nutrients from the plant material recently ingested and subsequently washed from the rumen. Thus many nutrients pass from the rumen in both an indigestible and a digestible form. Any measure of rumen digestion other than that of maximum digestion must, therefore, differentiate between these 2 types of passage.

A clear distinction between these 2 types of passage is dependent upon the degree of completion of the digestive processes within the 12-hour period following feeding. If rumen digestion is complete at 12 hours after feeding, the rumen contents present at that time would not undergo any further change and all digestion taking place during the next 12 hours would represent digestion of the newly ingested material. It is important, therefore, to examine evidence relating to the completeness of rumen action in the course of the normal 12-hour feeding interval. Hale, Duncan and Huffman ('40) removed rumen contents from cows at 14 and 24 hours after feeding. Rumen digestion coefficients as measured by lignin ratios were essentially unchanged, although half of the dry matter present in the rumen at 14 hours had passed from the rumen during the succeeding 10 hours. Excluding ether extract, which is complicated by a synthesis of fat, the highest value for rumen digestion observed for the 14-24-hour period was only 1.8%. This value is within the limits of experimental error. It might be suggested that even if further digestion had occurred a proportionate removal of lignin might obscure this digestion. Calculations of lignin digestion

[5] This refers to any concentration of the rumen contents irrespective of the digestion period and also includes the material reaching a maximum concentration in the current digestion period.

using formula (2) eliminate this possibility as zero values for lignin digestion were obtained at both 14 and 24 hours. A comparison of rumen concentration at 12 hours with fecal concentration gives further evidence of completed digestion. Under conditions at the Michigan Station average rumen coefficients at 12 hours for the dry matter in different lots of roughage varied from 45.7 to 48.7% while fecal coefficients for the same lots of roughage varied from 46.7 to 59.8%. Rumen concentration at 12 hours after feeding is thus seen to approach and, in some instances, to equal fecal concentration. The uniformity of the rumen values in contrast with the variable fecal values further suggests a "ceiling" on rumen digestion corresponding to the lowest fecal concentrations observed in digestion trials. All rumen digestion coefficients for 12 hours have approximated the level of such a hypothetical "ceiling" and suggest that the maximum limits of rumen digestion are normally reached within the 12-hour digestion period. The problem of a "ceiling" for rumen digestion is discussed further in the accompanying paper (Hale, Duncan and Huffman, '47). Miscellaneous observations of rumen physiology also suggest that the peak of digestion is reached within 12 hours after feeding. The rumen pH values begin to drop and reach a maximum acidity approximately 6 hours after feeding, after which time the values gradually increase to the prefeeding level (Monroe and Perkins, '39; Hale, Duncan and Huffman, '40; Myburgh and Quin, '43).

The microbiological studies of Bortree ('46) on the changes in rumen microflora from feeding might be applicable here. The conclusion that rumen digestion comes to an end prior to 12 hours after feeding would appear tenable. We may, therefore, proceed to a measurement of the different types of passage and to a calculation of rumen coefficients.

In general a digestion coefficient is calculated by dividing the amount of a nutrient digested by the amount of the same nutrient in the feed and multiplying the result by 100. The amount of a particular nutrient digested in the rumen, and

hence the rumen digestion coefficient can be determined on the basis of the following relationship:

$$\text{(a)}\quad \begin{matrix}\text{Amount of nutrient}\\ \text{digested in rumen}\end{matrix} = \left(\begin{matrix}\text{Total passage of the}\\ \text{nutrient from the rumen}\end{matrix} - \begin{matrix}\text{Passage of nutrient in}\\ \text{the undigested form}\end{matrix}\right)$$

Measurement of the total passage of dry matter, or of a particular nutrient, from the rumen can readily be accomplished by removing and weighing the entire contents of the rumen at the time of feeding and again at the end of the digestion period under investigation. The total nutrients in the rumen after feeding are represented by the sum of the rumen contents at feeding and the ingested hay. The amount of nutrients present at the end of the specified digestion period is subtracted from this total to give the total passage of nutrients from the rumen. As the total passage of nutrients from the rumen can thus be measured, the problem becomes one of measuring the passage of the concentrated rumen contents remaining from the previous digestion periods,[6] i.e., the passage of undigested nutrients.

Lignin values would seem readily adaptable for such a measurement. The percentage of lignin in the rumen contents at 12 hours also represents the percentage of lignin in the maximally concentrated rumen contents since digestion reaches a maximal level within the 12-hour period. The total amount of lignin passing from the rumen can be measured as above. If lignin leaves the rumen exclusively as a component of the maximally concentrated contents we may then calculate the total passage of these concentrated rumen contents as follows:

$$\text{(b)}\quad \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} = \begin{matrix}\text{Total amount of concentrated}\\ \text{contents passing from rumen}\end{matrix}$$

The amount of undigested nutrients passing from the rumen during the digestion period under investigation can be determined from formula (b).

A question might be raised as to whether or not lignin leaves the rumen exclusively as a component of the maximally

[6] Here again, this includes any concentrated rumen contents passing from the rumen, irrespective of the time that it was consumed.

concentrated rumen contents. As only small amounts of lignin are normally digested in the rumen (Hale, Duncan and Huffman, '47) and since such digestion can readily be corrected for by the use of formula (2), the possibility of lignin passing in a digested state does not present a problem. The only remaining path of passage would be as a component of plant fragments not yet subjected to the concentrating action of the rumen. The selective passage of highly comminuted material from the rumen as noted by Schalk and Amadon ('28), however, suggests that the passage of plant fragments not yet subjected to the concentrating action of the rumen would not be significant.

Formula (b) can be extended for a measurement of the passage of any particular nutrient in the undigested state since both the amount and composition of the concentrated rumen contents passing from the rumen can be determined. The calculation is represented as follows:

$$\text{(c)}\quad \left(\frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} \times \text{Per cent nutrient in rumen at 12 hours}\right) = \text{Amount of nutrient in undigested state}$$

The formula for measuring rumen digestion coefficients may now be expressed in the following completed form:

$$\text{(4)}\quad \frac{\left(\text{Total amount of nutrient leaving rumen} - \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin at 12 hours}}\right) \times \text{Per cent nutrient in rumen at 12 hours}}{\text{Amount of nutrient in hay}} \times 100 = \text{Digestion coefficient of nutrient}$$

This formula may be applied to studies of the extent of rumen digestion of any particular nutrient at any given period after feeding, thereby permitting an investigation of the rate of rumen digestion. The method proposed for calculating digestion coefficients for dry matter and protein at 6 hours after feeding is illustrated by the following examples.

Data.

1. Cow received 11.3 pounds of hay containing 15.2% lignin, 100% dry matter and 15.5% protein.

2. Rumen contents at feeding time (i.e., 12 hours after previous feeding) contained 25.2 pounds of dry matter, 28.9% lignin and 12.5% protein.

3. Rumen contents 6 hours after feeding contained 30.2 pounds of dry matter, 26.2% lignin and 12.8% protein.

The total passage of all nutrients (i.e., dry matter) and of lignin is needed to complete the values required in formula (4).

(a) Calculation of digestibility of dry matter:

$11.3 + 25.2 = 36.5$ lbs. of dry matter in rumen after feeding
$36.5 - 30.2 = 6.3$ lbs. of dry matter which left rumen in 6 hours
$25.2 \times 0.289 = 7.3$ lbs. of lignin in rumen before feeding
$11.3 \times 0.152 = 1.7$ lbs. of lignin in hay

Total = 9.0 lbs. of lignin in rumen after feeding

$30.2 \times 0.262 = 7.9$ lbs. of lignin in rumen at 6 hours

Difference = 1.1 lbs. of lignin which left rumen in 6 hours

Substitution in formula (4) gives

$$\frac{6.3 - \left(\frac{1.1 \times 1.00}{0.289}\right)}{11.3} \times 100 = 22.1\% \text{ dry matter digested.}$$

(b) Calculation of digestibility of protein:

$25.2 \times 0.125 = 3.2$ lbs. protein in rumen before feeding
$11.3 \times 0.155 = 1.8$ lbs. of protein in hay

Total = 5.0 lbs. of protein in rumen after feeding

$30.2 \times 0.128 = 3.9$ lbs. of protein in rumen at 6 hours

Difference = 1.1 lbs. of protein which left rumen in 6 hours

Substitution in formula (4) gives

$$\frac{1.1 - \left(\frac{1.1 \times 0.125}{0.289}\right)}{1.8} \times 100 = 33.3\% \text{ protein digested.}$$

Results obtained by applying the above method to studies of the rate of rumen digestion are presented in the accompanying paper (Hale, Duncan and Huffman, '47).

DISCUSSION

Recapitulation of methods. The theoretical and experimental basis for the calculation of rumen digestion coefficients

has been adequately discussed in the presentation of the methods and need not be commented on further.

In investigations of the fate of roughage in the rumen the investigator can employ either of 2 methods. In any study where a measure of the maximum digestion alone is desired formula (3) should be employed because of its greater simplicity and because only 1 removal of rumen contents is required. For investigations of the rate of rumen digestion formula (3) is inadequate and the investigator must apply formula (4) to obtain significant values. In this instance rumen contents are removed at the beginning of the digestion period under consideration and again at 12 hours after feeding. Since maximum digestion is achieved within 12 hours, both methods may be applied at or after that time with identical results.

Lignin values form the basis of both of these methods but are used in 2 distinct ways. In the first instance they are used to indicate the ratio between the lignin of the hay and that of the rumen contents thereby giving a measure of the concentration of the hay in the rumen. In the second instance lignin values are not used to measure concentration but rather to measure the amount of nutrients passing from the rumen in the digested and undigested form. The actual amounts are then used to calculate digestion coefficients. A method of measuring the rumen digestion of lignin is presented in formula (2) so that any discrepancies resulting from the digestion of lignin in the rumen may be eliminated.

These methods, while seemingly well adapted to studies with roughages, are not suitable to studies of mixed rations since concentrates are low in lignin and pass from the rumen more rapidly than roughages. It is suggested that rations containing concentrates may best be studied by use of the silk-sac method employed by McAnally ('42). The limitations of this method have been noted.

Problems of the chemical changes occurring in the rumen readily yield themselves to investigation by a combination of the methods described herein.

What is measured? Some question might arise as to whether these methods measure true digestion, i.e., the effective solution of nutrients, or a mere mechanical passage of plant particles which escaped the digestive processes. In view of the selective passage of highly comminuted material from the rumen (Schalk and Amadon, '28) and the tendency toward stagnation in the rumen, it is unlikely that any appreciable passage of plant particles not yet thoroughly subjected to the disintegrative action of the rumen would take place. This means that passage from the rumen is almost wholly in the form of either maximally concentrated rumen contents or of soluble nutrients. As the method presented herein differentiates between these 2 types of passage, it may be considered to measure an actual solution of nutrients.

Values for rumen digestion coefficients obtained by the methods presented are neither unduly high nor low (Hale, Duncan and Huffman, '47). They are well distributed over the entire range of possible values with nutrients of high digestibility approaching the upper limits and those of low digestibility nearing the lower. This condition is not likely to be obtained if any appreciable amount of plant material passed from the rumen before reaching the maximum state of digestion. Extremes observed were the zero digestion coefficient for crude fiber at 6 hours after feeding and the 65.2 and 83.0% digestion at 12 hours for nitrogen-free extract and other carbohydrates, respectively. The last 2 values represented 100.2 and 101.6% of the fecal concentration of these highly digestible nutrients, an unusually precise coincidence.

SUMMARY

Methods for calculating the maximum digestion in the rumen on the basis of lignin values have been extended to include techniques for measuring the rate of rumen digestion and the digestibility of lignin in the rumen.

Principles forming the basis for rumen digestion calculations receive special attention.

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RUMEN DIGESTION STUDIES

II. STUDIES IN THE CHEMISTRY OF RUMEN DIGESTION [1]

E. B. HALE,[2] C. W. DUNCAN AND C. F. HUFFMAN
Departments of Dairy and Agricultural Chemistry, and the Institute of Nutrition, Michigan State College, East Lansing, Michigan

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By applying new techniques to studies of rumen digestion of alfalfa hay in the bovine, Hale, Duncan and Huffman ('40) first demonstrated the magnitude of the chemical changes taking place in the rumen. Since that time the method of investigation has been extended to permit a study of the rate, as well as the magnitude, of these chemical changes (Hale, Duncan and Huffman, '47) and the results have been compared quantitatively with digestion in other portions of the digestive tract. The microbiological aspects of the chemical changes have been considered, with special emphasis on fat synthesis. The results obtained by applying these methods to studies on the rate of rumen digestion are presented in this paper.

METHODS

Rumen digestion coefficients were measured by the lignin-value method of Hale, Duncan and Huffman ('47). In all trials with the 2 rumen fistula cows the entire contents of the rumen were removed, weighed, mixed, sampled and returned immediately to the rumen after a representative sample of 7–10 pounds had been taken. Enough alcohol was added to give approximately a 50% concentration. The samples were stored

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[2] Present address, University of Chicago, Chicago, Illinois.

in the cold and subsequently dried in a current of warm air at 24°C. Samples were removed from cow R-4 at 6 and 12 hours and from animal R-1 at 14 and 24 hours after feeding. A period of from 1 to 2 weeks was allowed between such removals of rumen content to insure complete resumption of the normal function of the rumen.

In special studies of fat synthesis 3 cows were fed basic beet pulp rations low in fat. The specific rations are given in table 5. Representative samples were taken from the rumen of cow R-1 which was fed 2 different beet pulp rations. The 2 cows, C-167 and D-11, were slaughtered and representative samples of the rumen contents were taken immediately. These samples were taken to the laboratory and dried at once on the steam bath. Since lignin values are not applicable for measuring the concentration of the beet pulp rations in the rumen, the concentration was assumed to be 90% of the fecal concentration, a value suggested by studies of the rumen concentration of alfalfa. Morrison ('45) gives 71.8% for the fecal digestion of dried beet pulp. Ninety per cent of this value gives 64.6 as the maximum concentration likely to occur in the rumen. Under the conditions of this experiment, the theoretical increase of fat in the rumen can be calculated as follows when the beet pulp ration comprises 0.43% fat and the rumen contents actually contain 2.71% fat 8 hours after feeding (table 5):

$$(.0043 \times .646) \times 100 = 0.28\% \text{ increase of fat due to concentration}$$

$$0.43 + 0.28 = 0.71\% \text{ fat after concentration occurred (theoretical)}$$

$$\left(\frac{2.71 - 0.71}{0.71}\right) \times 100 = 281.7\% \text{ actual increase of fat in rumen}$$

Differences in the gross and net increases observed in table 5 emphasize the importance of eliminating increases due to the concentration of digestion when evaluating rumen synthesis in order to make the final test for fat synthesis more critical.

Lignin, cellulose and other carbohydrates were determined by the procedures of Crampton and Maynard ('38), fatty acids by the method of Horwitt, Cowgill and Mendel ('36), and all other nutrient values by the A.O.A.C. methods ('40).

RESULTS

Data relating to the removal of rumen contents are presented in table 1, and the percentage composition of the alfalfa hay and rumen contents is shown in table 2. Rumen digestion coefficients calculated at various periods subsequent to feeding are presented in table 3. Values for 12 hours or more after feeding were obtained by using formula (1) and those for less than 12 hours by formula (4) of Hale, Duncan and Huffman ('47). Fecal digestion was determined by the usual digestion trials. In all instances coefficients for the rumen digestion of lignin were calculated by using formula (2) of the above

TABLE 1

The amount and percentage of dry matter in the rumen.

HOURS AFTER FEEDING	DRY MATTER FED	TOTAL CONTENT OF RUMEN	DRY MATTER IN RUMEN	
	lbs.	*lbs.*	*%*	*lbs.*
		Cow no. R-4		
6	11.3	185	16.3	30.2
12	11.3	177	14.6	25.8
6	11.3	185	16.2	30.0
12	11.3	174	14.2	24.7
		Cow no. R-1		
14	8.8	132	14.2	18.7
24	8.8	65	14.5	9.4

TABLE 2

Percentage composition of alfalfa hay and rumen contents on the dry basis.

NUTRIENT	COW NO. R-4			COW NO. R-1			AV. OF 8 TRIALS	
	Alfalfa hay	Rumen		Alfalfa hay	Rumen		Alfalfa hay	Rumen
		6 hr.	12 hr.		14 hr.	24 hr.		12–14 hr.
Protein	15.5	12.8	12.5	17.0	11.8	11.0	16.6	13.0
Ether extract	1.6	2.5	2.1	1.3	1.9	1.5	2.0	2.5
Crude fiber	31.4	43.6	44.8	38.1	52.0	51.5	33.3	47.0
N-F extract	45.1	31.9	31.5	36.9	24.7	24.6	41.1	27.7
Cellulose	34.7	37.0	36.7	31.3	37.8	34.6	33.6	36.8
Other CHO	26.6	12.3	10.7	26.1	4.0	8.5	24.9	8.2
Lignin	15.2	26.2	28.9	17.5	34.8	33.0	16.1	30.2
Dry matter, lbs.	11.3	30.2	25.2	8.8	18.7	9.4	...	...

authors. Rumen coefficients corrected for lignin digestion are given in column 7 of table 3 and were obtained by using formula (3) of Hale, Duncan and Huffman ('47). The rumen coefficients at 12, 14 and 24 hours were not in need of correction as lignin was not observed to be digested in those trials. At 6 hours, 3.7% lignin was observed to be digested but since the value at 12 hours for the same lot of hay and the same animal was —0.4% the first value was considered to be largely an experimental error and corrections were not made. The average rumen coefficients for 8 trials at 12 to 14 hours after

TABLE 3

Rumen and fecal digestion coefficients.

NUTRIENT	COW NO. R-4			COW NO. R-1		AV. OF 8 TRIALS		
	Rumen		Fecal	Rumen		Rumen		Fecal
	6 hr.	12 hr.		14 hr.	24 hr.	12–14 hr.	Corrected [1]	
Dry matter	22.1	47.8	59.8	50.0	46.6	47.1	48.4	57.0
Protein	33.3	61.1	69.7	66.7	66.7	58.6	59.6	69.1
Ether extract	—72.2	33.3	43.2	26.3	38.6	32.5	35.4	19.3
Crude fiber	—0.3	25.7	49.6	32.4	29.4	25.5	27.2	46.9
N-F extract	45.1	62.7	68.5	65.6	62.5	64.6	65.2	65.1
Cellulose	12.8	43.6	63.1	39.3	42.9	42.1	43.4	55.0
Other CHO	53.3	80.0	74.7	91.3	82.6	81.8	83.0	81.7
Lignin	3.7	—0.4	32.7	0.0	0.0	3.1	3.1	21.5

[1] Corrected for lignin digestion.

feeding include data previously reported by Hale, Duncan and Huffman ('40). This number of trials should establish the maximum digestion of alfalfa hay in the rumen with some degree of certainty. A more graphic presentation of the rate of rumen digestion and of digestion in the remainder of the digestive tract is given in table 4. Data for cow R-4 were used to calculate digestion coefficients for the first and second 6-hour periods and data for cow R-1 to calculate the coefficients for the second 12-hour periods. Average rumen and fecal coefficients for the 8 trials were used in computing digestion coefficients after passage of material from the rumen.

Dry matter was digested at an even rate throughout the 12-hour digestion period with an average digestion of 48.4%

obtained at 12–14 hours. Protein, nitrogen-free extract and other carbohydrates were very rapidly removed during the first 6 hours. During the second 6 hours these nutrients were removed at approximately the same rate as the other nutrients. Average coefficients at the 12–14-hour period were 59.6, 65.2 and 83.0%, respectively. Crude fiber was not digested during the first 6 hours but the average coefficient at 12–14 hours was 27.2%. Apparent lignin digestion was only 3.1% at the 12–14-hour period (table 3). Cellulose was digested largely during the second 6-hour period when 30.8% was digested as contrasted with 12.8% digested during the first 6 hours (table 4).

TABLE 4

The disintegration of alfalfa hay in the ruminant digestive tract.

NUTRIENT	RUMEN			DIGESTION AFTER LEAVING RUMEN [3]	RUMEN DIGESTION AS PER CENT OF FECAL DIGESTION [4]
	1st 6 hr.	2nd 6 hr.[1]	2nd 12 hr.[2]		
	%	%	%	%	%
Dry matter	22.1	25.7	—3.4	8.6	84.9
Protein	33.3	27.8	0.0	9.5	86.3
Ether extract	—72.2	105.5	12.3	—16.1	
Crude fiber	—0.3	26.0	—3.0	19.7	58.0
N-F extract	45.1	17.6	—3.1	—0.1	100.2
Cellulose	12.8	30.8	3.6	11.6	78.9
Other CHO	53.3	26.7	—8.7	—1.3	101.6
Lignin	3.7	—4.1	0.0	18.4	14.4

[1] Difference between columns 1 and 2 in table 3.
[2] Difference between columns 4 and 5 in table 3.
[3] Difference between columns 7 and 8 in table 3.
[4] Obtained from columns 7 and 8 in table 3.

When removal of the rumen contents was delayed until 24 hours after feeding, the highest increase in digestion, excluding ether extract which is complicated by fat synthesis, was only 3.6% (table 4). This slight digestion of cellulose during the second 12 hours is in marked contrast to the great activity of the first 12 hours. The digestion of lignin after 24 hours in the rumen was observed to be zero in this instance.

After the plant fragments passed from the rumen an average of 8.6% of the dry matter was digested (table 4). Nitro-

gen-free extract and other carbohydrates were not further digested after leaving the rumen. Of the other nutrients protein averaged 9.5, lignin 18.4, cellulose 11.6, and crude fiber 19.7% digestibility in the lower digestive tract. The average lignin digestion of 18.4% is in marked contrast to the average rumen value of 3.1%. Lignin was the only nutrient digested to a greater extent after passing from the rumen than while in the rumen.

TABLE 5

Increase in the ether extract content of the rumen over that in the ration.

COW NO.	RATION	TIME AFTER FEEDING	ETHER EXTRACT IN RATION	ETHER EXTRACT IN RUMEN	GROSS INCREASE	NET INCREASE [1]
		hrs.	%	%	%	%
R-4	25 lbs. alfalfa	6	1.62	2.46	51.7	54.9
R-4	25 lbs. alfalfa	12	1.62	2.14	32.1	—28.6
R-1	30 lbs. alfalfa	14	0.50	1.10	120.0	26.3 [2]
R-1	20 lbs. alfalfa	14	0.69	0.83	20.6	—33.2 [2]
R-1	Beet pulp + C [3]	10	0.25	1.10	340.0	169.0 [2]
R-1	Beet pulp + M + S [4]	8	0.43	0.66	53.5	—7.0
C-167	Beet pulp + M + S [4]	8	0.43	1.39	223.3	95.8
D-11	Beet pulp + M + S [4]	8	0.43	2.71	530.2	281.7

[1] After increase due to concentration of digestion was eliminated.
[2] Fatty acid values used. All others are ether extract values.
[3] 12 lbs. beet pulp plus 0.25 lbs. casein.
[4] 10 lbs. beet pulp plus 5 lbs. molasses plus 2 lbs. starch.

When ether extract values were used with an alfalfa hay ration, synthesis was observed at the height of digestion only, i.e., 6 hours after feeding when an increase of 54.9% was noted. A summary of the data on fat synthesis is presented in table 5. During the second 6 hours there was a rapid dissipation of the accumulated fat which carried over into the second 12-hour period (table 4). When fatty acid values were used a synthesis was observed at 14 hours after feeding on a 30-pound hay ration but when the ration was reduced to 20 pounds per day a negative digestion coefficient of 33.2% was noted. Net increases in fat on the beet pulp ration varied from —7.0 to 281.7%. These variations occurred independently of the added

supplements. The highest value, 281.7%, was obtained from ether extract determinations. A high value of 169.0% was observed when fatty acid values were used.

DISCUSSION

Although many details of the basic chemical changes occurring in the rumen are as yet missing and stand in need of further investigation, sufficient data have been accumulated to permit a consideration of some of the salient features.

Dry matter was digested at an even rate throughout the 12-hour digestion period. During the first 6 hours the predominant phenomenon was the rapid disappearance of the more soluble nutrients, proteins and carbohydrates, from the rumen. Cellulose was only slightly digested and lignin and crude fiber escaped disintegration. The slow rate of digestion of cellulose during this period suggests that bacterial action was not extensive. However, Baker ('39) observed that hemicellulose and pectic substances are readily attacked by the iodophile microflora of the rumen and bacterial action of this nature may be responsible for much of the carbohydrate digestion observed. Whether the microorganisms play a role in the metabolism of the protein which was so rapidly removed from the plant skeleton is not clear.

During the second 6 hours after feeding cellulose was rapidly disintegrated. The digestion of both proteins and carbohydrates paralleled the digestion of cellulose. Digestion of lignin in the rumen never exceeded more than a few per cent. The marked contrast between the fate of lignin in the rumen and in other parts of the digestive tract suggests that functionally the rumen is not a factor in the degradation of lignin. This point is particularly supported by the observation that although the fecal digestion of lignin in the hay fed cow R-4 was 32.7%, the digestion of lignin in the rumen 12 hours after feeding was zero. The rapid removal of cellulose during the second 6-hour period indicates that the cytoplastic processes of the iodophile microorganisms described by Baker ('42a) and observed by Bortree ('46) are likely to be the pre-

dominant phenomena throughout this period. By employing polariscopic techniques Baker ('39) and Bortree ('46) have observed that the contours of the area of disintegration of plant fragments removed from the rumen correspond precisely with the location of the microorganisms. This observation is highly indicative that the observed organisms are the effective agents of disintegration in the rumen. Rumen microorganisms are apparently capable of digesting widely varying amounts of roughage with equal efficiency since Hale, Duncan and Huffman ('40) did not observe any differences in the extent of rumen digestion of alfalfa hay when fed at levels varying from 10 to 30 pounds per day.

Rumen digestion was observed to come to a standstill within 12 hours after feeding and a maximum level of digestion was always obtained at this time. No significant increase in digestion coefficients was observed when the removal of rumen contents was delayed until 24 hours after feeding instead of 12 hours.

The marked decrease in rumen digestion during the second 12-hour period is in contrast to the high rate of digestion observed during the first 12-hour period. Rumen digestion coefficients for the various lots of hay used in these studies varied from 45.7 to 48.7% while fecal coefficients for the same lots of roughage varied from 46.7 to as much as 59.8%. Rumen coefficients thus appear to remain constant, irrespective of the fecal digestibility of the hay. This suggests a "ceiling" on rumen digestion, corresponding to the lowest level of fecal digestion usually observed in digestion trials. The probable role of lignin in imposing this "ceiling" on rumen digestion has been suggested by digestion trials at this station (Hale, Duncan and Huffman, '47). The fecal digestion of lignin was observed to vary from zero to as high as 47%. When the digestibility of lignin was zero, fecal digestion for dry matter corresponded to the average rumen digestion of dry matter. When fecal digestion of lignin was noted, the fecal digestion of dry matter was observed to exceed rumen digestion coefficients in direct ratio to the amount of lignin digested.

Evidence is thus obtained that the chemical composition of the plant material imposes certain limitations upon the activity of rumen microflora. The data suggest that lignin, which is relatively indigestible in the rumen, is important in imposing a limit on the rumen digestion of roughages. In this connection Baker and Martin ('37) and Baker ('39) reported that highly lignified tissues remained intact while slightly lignified tissues rich in hemicellulose were rapidly disorganized. Cutin and suberin were not attacked by the iodophile bacteria and therefore conferred immunity to the structure in which they were incorporated.

After the plant fragments pass from the rumen lignin is digested to a variable extent. This digestion is most likely produced by the acid content of the abomasum, since lignin is known to be partially soluble in dilute acid solution (Harris and Mitchell, '39), as well as some enzyme present in the gastric juice (Csonka, Phillips and Jones, '29). In any event the variable digestion of lignin after its passage from the rumen exposes cellulose and protein to further decomposition in the caecum. Under the conditions of these experiments an average of 11.6% cellulose and 9.5% protein was released in this manner after passing from the rumen. This digestion is most probably brought about by the iodophile microflora of the caecum and herein lies the major function of that organ in the ruminant animal. As this digestion is quite variable and usually not very extensive, the caecum can be considered to act only in a supplementary capacity to the rumen. Regarding the function of the caecum in ruminants Baker ('39) states, "Thus, whereas in the caecum of nonruminants the disintegration of cellulose is actively in progress, in the caecum of ruminants the changes taking place in the rumen have long since come to a standstill." He further observed that plant fragments in the caecum are in an advanced stage of disintegration but that the iodophile microorganisms so conspicuous upon them in the rumen are, for the most part, absent from the still visible areas of erosion.

In contrast to the rapid rate of rumen digestion reported in this paper, McAnally ('42) found that several days were required to obtain any considerable digestion in sacs suspended in the rumen. The causes of the impeded digestion are not altogether clear but it is probable that the lack of direct mastication, salivation and rumination was responsible. This suggests that these physical factors acting upon ingesta before it leaves the rumen serve to accelerate the rate of rumen digestion. Baker and Martin ('37) noted that gross fracture of the plant structures facilitated entrance of microorganisms. The degree to which such relationships are effective needs further clarification.

Rumen synthesis of fatty acids was demonstrated with both alfalfa hay and beet pulp rations. Since the net increases shown in table 5 do not include the increases resulting from the concentration of digestion, these values are considered especially significant. The observation that synthesized fat is rapidly dissipated from the rumen together with calculated increases approaching 300% indicates that fat synthesis may be sufficient to make a highly significant contribution to the nutrition of the ruminant. Whether or not the fatty acids observed in the rumen are intermediate or end products of digestion is not clear. Baker ('39) found that the iodophile microflora of the rumen not only digest cellulose but also act as acceptors for the products of digestion. He concluded provisionally (Baker, '42b) that materials synthesized in the rumen, such as polysaccharides, rather than the initial products of digestion, such as organic acids, are utilized by the host animal. Van der Wath and Myburgh ('41) are of the opinion that iodophile bacteria are able to synthesize glycogen within their bodies by utilizing the products of digestion. It may be, therefore, that the increases in fatty acids observed herein were due to an accumulation of the initial products of digestion not yet synthesized to polysaccharides by the microflora. Synthesis of the fatty acids to polysaccharides could further account for the rapid dissipation of the fatty acids from the rumen. On the other hand, the fatty acids may be utilized by

the animal as such or may represent products of bacterial metabolism. Controlled microbiological studies are necessary for the resolution of this problem. The nature of the fatty acids present in the rumen was not investigated.

SUMMARY

Studies of the chemical changes in the rumen suggest the following salient features regarding the fate of roughage in the ruminant digestive tract:

1. During the first 6 hours after feeding the predominant phenomenon was the rapid digestion of proteins and carbohydrates. Decomposition of cellulose began during this period but was not extensive.

2. The predominant phenomenon during the second 6 hours was the rapid disintegration of cellulose. Digestion of proteins and carbohydrates in this period paralleled the digestion of cellulose. Although small amounts of lignin may be attacked in the rumen, functionally the rumen is not a factor in lignin digestion.

3. Rumen digestion was observed to come to a standstill within 12 hours after feeding. Prolonging the digestion periods to 24 hours did not increase rumen digestion coefficients. Lignin apparently protects plant fragments from further action by rumen microflora.

4. Average rumen digestion coefficients for 8 trials at 12–14 hours after feeding were: dry matter 48.4, protein 59.6, nitrogen-free extract 65.2, crude fiber 27.2, cellulose 43.4, other carbohydrates 83.0 and lignin 3.1%.

5. After plant fragments pass from the rumen variable amounts of lignin may be digested, thereby exposing varying amounts of cellulose and protein to further digestion. Fecal digestion was observed to be quite variable even though rumen digestion of the same lots of hay was very constant.

6. The caecum plays only a supplementary role in the disintegration of roughage within the ruminant organism. An average of 11.6% of the cellulose and possibly 9.5% protein was observed to be digested in the caecum.

7. The production of fatty acids in the rumen was demonstrated. Maximum increases, after the increase due to digestion was eliminated, were 54.9% on an alfalfa hay ration and 281.7% on a beet pulp ration.

8. The rapid dissipation of fatty acids from the rumen together with the marked increases noted suggests that fatty acids may make a highly significant contribution to the nutrition of the ruminant either as an intermediate or end product of digestion.

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